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<b>Applicant</b> RUELE, Jean-Louis	

1. The designated Office is hereby notified of its election made:

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30 June 2000 (30.06.00)

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Dear Examiner,

The attached search was run with the most recently released version of Compugen's search software, GenCore 5. With this update, several changes have occurred in the results of FrameSearches (protein query sequence vs nucleic acid databases or nucleic acid query sequence vs protein databases).

**In reference to FrameSearches:**

- The output format has been improved so that it more closely resembles the format for standard search output.
- Calculation of Percent Similarity has been changed for FrameSearches. The new method of calculation is more similar to the method used in NCBI's BLAST algorithm. The same results are found in the same order using GenCore 5 and the previous version of GenCore, but Percent Similarities are lower in GenCore 5 results.
- The formula for % similarity calculation is:

$$100 * \frac{\text{matches} + \text{conservative substitutions}}{\text{alignment\_length}}$$

where "matches" is the number of identical matches and "conservative substitutions" is the number of non-identical positive matches.

- GenCore 4.5 considers the match Thr vs GCT (Ala) to be a similarity since BLOSUM62 gives score of 0 to this match. It is marked by ':::' in the alignment:

```
Qy    46 AspSerThrAspAla.Met..Gly 52
      |||||::: ||| ::: |||
Db    605 GATTCCGCTGCTGCTAATTTTGGC 628
```

GenCore 5 requires a positive score to consider a non-identical match a similarity, therefore the same 'match' is not emphasized in the new alignment:

```
Qy    46 AspSerThrAspAla.Met..Gly 52
      ||||| ||| ::: |||
Db    605 GATTCCGCTGCTGCTAATTTTGGC 628
```

-If you have any questions, please feel free to contact one of the searchers in Biotech/Chem Library.



**Set Name Query**

side by side

**Hit Count Set Name**

result set

*DB=USPT,JPAB,EPAB,DWPI; PLUR=YES; OP=ADJ*

<u>L15</u>	L14 and (carrier or oil-in-water? or aluminum)	40	<u>L15</u>
<u>L14</u>	L13 and (3d mpl or q21)	48	<u>L14</u>
<u>L13</u>	L11 and(th1 or t cell respon?)	938	<u>L13</u>
<u>L12</u>	L11 and TH1 type?	0	<u>L12</u>
<u>L11</u>	adjuvant	64493	<u>L11</u>
<u>L10</u>	L7 and ( stabilize )	169	<u>L10</u>
<u>L9</u>	L8 and(t-helper epitope)	8	<u>L9</u>
<u>L8</u>	L7 and ( stabilize )	169	<u>L8</u>
<u>L7</u>	recombinant fusion protein and( t-helper epitopes or facilitate or stabiliz)	876	<u>L7</u>
<u>L6</u>	L5 and l2	681	<u>L6</u>
<u>L5</u>	L1 and (recombinant)	25135	<u>L5</u>
<u>L4</u>	L3 and moraxella	8	<u>L4</u>
<u>L3</u>	L2 and ( t-helper epitopes or facilitate? or stabiliz?)	349	<u>L3</u>
<u>L2</u>	L1 and isolated peptide	744	<u>L2</u>

*DB=DWPI,USPT,EPAB,JPAB; PLUR=YES; OP=ADJ*

<u>L1</u>	fusion or chimeric pro?	130733	<u>L1</u>
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END OF SEARCH HISTORY



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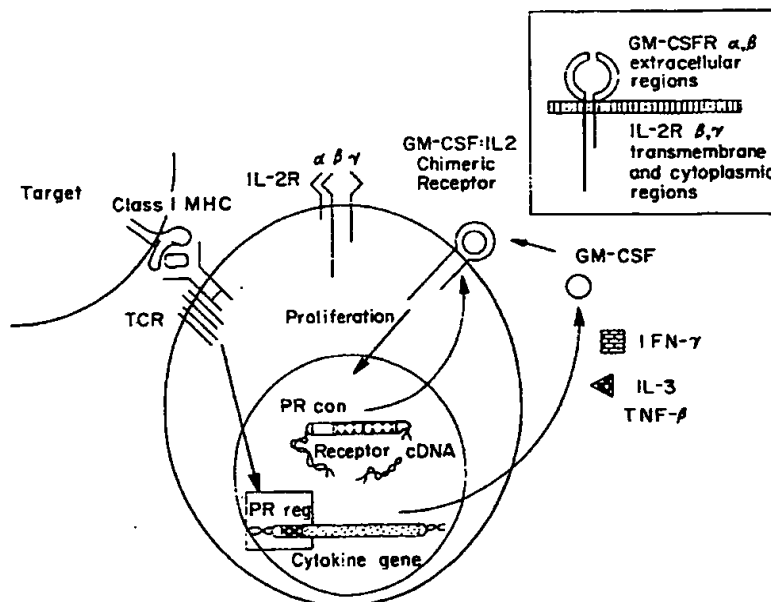
(74) Agent: DYLAN, Tyler, Morrison & Foerster, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

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*With international search report.*

**(54) Title: CHIMERIC CYTOKINE RECEPTORS IN LYMPHOCYTES**



Recombinant polynucleotides are provided that encode chimeric cytokine receptors. The chimeric receptor is comprised of an extracellular domain derived from cytokine receptor A-R that binds cytokine A, fused to a transmembrane and cytoplasmic domain derived from cytokine receptor B-R that binds cytokine B. The figure depicts a chimeric receptor with a GM:GSF receptor extracellular domain fused to an IL 2 receptor transmembrane and cytoplasmic domain. When the chimeric receptor is expressed in a lymphocyte it lessens the growth dependency of the lymphocyte on cytokine B in the presence of cytokine A.





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## CHIMERIC CYTOKINE RECEPTORS IN LYMPHOCYTES

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### Technical Field

The invention relates to the field of immunotherapy, more specifically to the introduction of genetic material encoding chimeric cytokine receptors into lymphocytes to reduce the dependency of the activated lymphocytes on T helper cells and/or growth factors supplied by T<sub>H</sub>-cells.

### Background

T lymphocytes are responsible primarily for protection against intracellular pathogens and malignant tumors. Individuals who are grossly deficient in T-cell immunity frequently succumb to overwhelming infections by organisms such as cytomegalovirus, *Pneumocystis carinii*, *Candida*, and other apparently "opportunistic" pathogens, including bacteria, viruses and fungi. Immunosuppression can result from a variety of causes, including viral infections (for example, with the HIV virus), as a result of chemical therapy, and malignancies (particularly of types that affect the hematopoietic system). T-cell immunity is also the major mechanism for rejection of allogeneic tissue or organ transplants. In fact, a major limitation to transplant therapy has been the difficulty in suppressing T-cell allograft rejection reactions without overly compromising vital protective mechanisms.

The adoptive transfer of antigen (Ag)-specific T cells to establish immunity appears to be an effective therapy for some viral infections and tumors in the mouse animal model system. (For a review, See P.D. Greenberg, in *Advances in Immunology* F. Dixon Ed. Academic Press, Inc. Orlando Fla. (1991), pp. 280-355.) However, the efficacy of an adoptive transfer method is dependent upon many factors, including the longevity of the transferred clones and the lack of toxicity to the host of the transferred cells.



HIT<sub>C</sub> specific for a retrovirally transformed tumor have been shown to eradicate the tumor cells and persist long-term in vivo following their engraftment (Klarnet et al., 1989, supra). However, analogous human HIT<sub>C</sub> cells having specificity for many important antigens, such as HIV, have not yet been isolated.

5 Realizing the full potential of antigen-specific T cells in therapy would be facilitated by developing a more complete repertoire of lymphocytes, in particular CTLs, with a lessened dependency on T<sub>H</sub>-cells. One approach has been the introduction of a recombinant vector that expresses a cytokine receptor, for example the IL-1 receptor, into T<sub>H</sub>-dependent CTL, resulting in the conversion to CTLs with a lessened dependency on IL-2. (PCT/US91/06921, WO  
10 92/05794, published April 16, 1992). The present invention, described below, presents a different approach to the production of lymphocytes with lessened dependency on one or more factors that stimulate growth and proliferation. As a part of the invention, lymphocytes with a lessened dependency on a stimulatory factor are produced via the expression of chimeric cytokine receptors that enable the cells to proliferate despite limiting quantities of a normally  
15 required cytokine, for example IL-2. The chimeric receptors contain domains of cytokine receptor chains, for example, IL-2 receptor (IL-2R), fused to domains of heterologous cytokine receptors. Chimeric receptors containing a portion of the IL-2R have been reported. In one chimeric receptor construct, an extracellular domain of IL-2R (now known to correspond to the  $\alpha$ -chain) was fused to a transmembrane and intracellular tyrosine kinase domain of epidermal  
20 growth factor receptor (EGFR). The polypeptide was expressed in fibroblasts, yielding a product which reportedly had high affinity for IL-2, and which reportedly transformed the fibroblasts. Bernard et al. (1987), Proc. Natl. Acad. Sci. USA 84:2125-2129. However, it has been reported that fibroblastoid cell lines do not to respond to IL-2 by proliferation. (Cf. Takeshita et al. (1992), Science 257:379-382, citing S. Minamoto et al. (1990), J. Immunol.  
25 145:2177). In another chimeric construct, the extracellular domain of IL-2R  $\beta$ -chain was fused to the cytoplasmic domain of murine erythropoietin receptor (EPO-R). The resultant construct was reportedly not capable of transducing an IL-2 induced signal. Mori et al. (1991), Int. Immunol. 3:149-156.

### 30 Summary of the Invention

The invention provides polynucleotide constructs encoding chimeric receptors that enable activated lymphocytes, particularly cytotoxic T lymphocytes (CTLs), to proliferate in response to an alternative cytokine other than one that is normally required. Preferably the alternative cytokine is one that is synthesized within the lymphocyte in response to antigen  
35 stimulation, which would thereby provide the lymphocyte with regulated autocrine growth.



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Mature T lymphocytes generally express the CD3 cell surface molecule, but consist predominantly of two basic subtypes based on their mutually exclusive expression of cell surface molecules CD4 and CD8. CD4<sup>+</sup> T cells are generally involved in "helper" functions in immune responses and secrete cytokine molecules, in particular interleukin 2 (IL-2), upon which the cytotoxic CD8<sup>+</sup> T cells are dependent. CD4<sup>+</sup> T cells are often referred to as T helper (T<sub>H</sub>) cells. CD8<sup>+</sup> cells are involved in "effector" functions in immune responses, such as direct cytotoxic destruction of target cells bearing foreign antigens, and represent an important mechanism for resistance to viral infections and tumors. The functional distinction between CD4<sup>+</sup> and CD8<sup>+</sup> T cells is based on the ability of CD4<sup>+</sup> cells to recognize antigen presented in association with class II MHC molecules, and CD8<sup>+</sup> cells to recognize antigen presented in association with class I MHC molecules. The CD8<sup>+</sup> cells that mediate this lytic function are designated cytotoxic T lymphocytes (CTLs). Although most CTL are of the CD8<sup>+</sup> phenotype, some CTL of the CD4<sup>+</sup> phenotype have been described. Generally, individual CTLs (whether CD8<sup>+</sup> or CD4<sup>+</sup>) are antigen-specific.

Lymphocytes are dependent upon a number of cytokines for proliferation. For example, CTLs are dependent on helper T (T<sub>H</sub>) cell-derived cytokines, such as IL-2, for growth and proliferation in response to foreign antigens. (Zinkernagel and Doherty, Adv. Immunol. 27:51, 1979; Male et al., Advanced Immunology, Chap. 7, Gower Publ., London, 1987; Jacobson et al., J. Immunol. 133:754, 1984). IL-2, for example, is a potent mitogen for cytotoxic T lymphocytes. (Gillis and Smith, Nature 268:154, 1977), and the combination of antigen and IL-2 causes proliferation of primary CD8<sup>+</sup> T cells in vitro. The importance of IL-2 for the growth and maintenance of the CD8<sup>+</sup> CTL in vivo has been documented in models of adoptive immunotherapy in which the therapeutic efficacy of transferred anti-retroviral CD8<sup>+</sup> cells is enhanced by subsequent administration of IL-2 (Cheever et al., J. Exp. Med. 155:968, 1982; Reddehase et al., J. Virol. 61:3102, 1987). IL-4 and IL-7 are also capable of stimulating the proliferation of at least a sub-population of mature CD8<sup>+</sup> CTL (Alderson et al., J. Exp. Med. 172:577, 1990).

Due to the specificity of T cells for "non-self" antigens, considerable research has been focused on the use of T cells in treating viral infections and malignant tumors. Cytotoxic T cells specific for a particular type of tumor antigen can be isolated and administered to a patient having the tumor, with the effect that the CTLs ameliorate the tumor. It has been demonstrated, for example, that tumor-specific T cells cannot only be generated to experimental tumors in mice, but also that T cells with apparent tumor specificity can be isolated from cancer patients. Such human tumor infiltrating lymphocytes (TILs) have been expanded in vitro and used to treat cancer patients, generating significant enthusiasm for human adoptive





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immunotherapy with tumor-specific T cells (Rosenberg et al., N. Engl. J. Med. 319:1767, 1988).

Similar studies using cytotoxic T cells specific for viral antigens have also been conducted in animal models. Human HIV-specific CTL of both the CD8<sup>+</sup> (Walker et al., Nature 328:345, 1987; Plata et al., Nature 328:348, 1987) and CD4<sup>+</sup> (Siliciano et al., Cell 54:561, 1988) phenotype have been isolated and characterized. HIV-specific CD8<sup>+</sup> CTL are classical CTL in that their cytotoxic responses are antigen-specific and MHC-restricted (Walker et al., supra; Chenciner et al., Eur. J. Immuno. 19:1537, 1989; Walker et al., Proc. Natl. Acad. Sci. USA 86:9514, 1989), in common with the numerous mouse and human CTL clones which have been characterized which are specific for viral, tumor or allospecific antigens.

Although many antigen-specific T cell clones have been isolated, the use of tumor-specific T cell clones generated in vitro has been shown to have definite limitations in tumor therapy. It has been demonstrated in several therapeutic models that the efficacy of cytolytic CD8<sup>+</sup> T cells is limited by a dependency on exogenous IL-2 (normally produced by T<sub>H</sub> cells), a finding that has been substantiated in human adoptive therapy trials in which administration of exogenous IL-2 appears essential for optimal therapeutic efficacy (Rosenberg et al., N. Engl. J. Med. 319:1767, 1988; Klarnet et al., in Role of Interleukin-2 Activated Killer Cells in Cancer, Lutzova and Herberman (eds.), CRC Press, Florida, Chap. 14, pp. 199-218, 1990). Thus, while in vitro T cell cloning techniques provide a means to generate large numbers of T cells with demonstrable tumor or viral specificity, the full potential of using such antigen-specific T cells in therapy appears to be limited by their dependency on cytokines normally produced by T<sub>H</sub> cells.

In some limited instances the problem of T<sub>H</sub> dependency may be circumvented by using a particular class of cells known to function independent of T<sub>H</sub> cells. These cells are known as helper-independent cytolytic CD8<sup>+</sup> cells (HIT<sub>C</sub>) (Klarnet et al., J. Immunol. 142:2187, 1989) and have been identified in populations of primary (i.e., freshly isolated from in vivo sources) CD8<sup>+</sup> CTL (Sprent and Schaefer, J. Exp. Med. 162:21068, 1985; Andrus et al., J. Exp. Med. 159:647, 1984). HIT<sub>C</sub> cells produce sufficient IL-2 to grow independently of CD4<sup>+</sup> cells and the cytokines they produce. HIT<sub>C</sub> cells have been shown to express plasma membrane IL-1 receptors (IL-1R) and require IL-1 for their IL-2-independent proliferation (Klarnet et al., 1989, supra). This is in contrast to conventional CD8<sup>+</sup> CTL which do not express detectable IL-1R on their surface (Lowenthal and MacDonald, 1987). HIT<sub>C</sub> cells have been generated which are specific for a range of antigens, including tumor, viral and alloantigens (von Boehmer et al., J. Immunol. 133:59, 1984; Klarnet et al., J. Immunol. 138:4012, 1987; and Andrus et al., J. Exp. Med. 149:647, 1984; Mizouchi et al., J. Immunol. 142:270, 1989).

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The chimeric constructs expressed from the polynucleotides contain a cytoplasmic region derived from the receptor of the normally required cytokine, for example IL-2R, joined, via a transmembrane domain, to an extracellular domain derived from the alternative cytokine receptor (e.g., GM-CSF-R). These chimera bind the cytokine (e.g., GM-CSF) recognized by the extracellular region of the alternative cytokine receptor (e.g. GM-CSF-R), and transmit the signal that generally occurs when the normal cytokine (e.g., IL-2) binds to its receptor (e.g. IL-2R), resulting in proliferation. The invention also includes the use of the polynucleotides encoding the chimeric receptors to prepare lymphocytes with lessened dependency on a cytokine, and the cells containing the chimeric constructs. The use in immunotherapy of lymphocytes containing polynucleotides encoding the chimeric receptors is also within the scope of the invention.

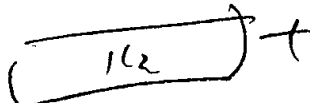
Accordingly, one embodiment of the invention is a chimeric receptor comprising one or more chimeric peptide chains that have an extracellular domain derived from cytokine receptor A-R that binds cytokine A, joined, via a transmembrane domain, to a cytoplasmic domain derived from cytokine receptor B-R that binds cytokine B, wherein cytokine B is a cytokine that is normally required by a lymphocyte for growth and proliferation and wherein the chimeric receptor expressed in said lymphocyte lessens the growth dependency of the lymphocyte on cytokine B in the presence of cytokine A.

Another embodiment of the invention is a recombinant polynucleotide encoding one or more of the above-described peptide chains. Yet another embodiment is a cell containing such a recombinant polynucleotide. The recombinant polynucleotide may be in the form of a recombinant expression vector. The cell may be a lymphocyte.

Still another embodiment of the invention is a method of using a recombinant polynucleotide comprising a region encoding a first peptide chain, wherein the first peptide chain comprises an extracellular domain derived from cytokine receptor A-R that binds cytokine A, joined, via a transmembrane domain, to a cytoplasmic domain derived from cytokine receptor B-R that binds cytokine B, wherein cytokine B is a cytokine that is normally required by a lymphocyte for growth and proliferation and wherein the chimeric receptor expressed in said lymphocyte lessens the growth dependency of the lymphocyte on cytokine B in the presence of cytokine A, the method comprising transforming a cell with the recombinant polynucleotide.

Yet another embodiment of the invention is a cell produced by the above-described method, and progeny thereof.

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### Brief Description of the Drawings

Figure 1 is an illustration of a prototypic chimeric receptor that can be employed in the overall strategy of the present invention.

Figure 2 is an illustration of a prototypic chimeric receptor in which the non-IL-2R  
5 extracellular domain is from a cytokine receptor that contains a single type chain, c-kit, attached to two different IL-2-derived intracellular domains.

Figure 3 is a graph showing a trace of fluorescent scanning for surface expression of transfected receptors on CTLL2 cells.

Figure 4 is a graph showing the effect of ligands on the proliferation of CTLL2 cells  
10 transfected with c-kit/IL-2R chimeras.

Figure 5 is a schematic illustration of chimeric GM-CSF/IL-2 receptors. In the upper left, the extracellular region of the human GM-CSF-R  $\alpha$  or  $\beta$  chains and the intracellular regions of human IL-2R $\beta$  or IL-2R $\gamma$  chains were used to construct chimeric receptors designated GM $\beta$ /2 $\beta$  and GM $\alpha$ /2 $\gamma$ , respectively (shown in the upper right); which undergo  
15 heterodimerization upon binding GM-CSF (shown in the lower portion of Figure 5).

Figure 6 is a graph showing IL-2- and GM-CSF-induced proliferative responses of the parental (CTLL2) T cell line and of transfectants expressing GM $\beta$ /2 $\beta$  or GM $\alpha$ /2 $\gamma$ , or co-expressing GM $\beta$ /2 $\beta$  and GM $\alpha$ /2 $\gamma$ .

### Detailed Description of the Invention

The invention provides antigen specific lymphocytes that differ from the parental lymphocytes by the presence of a recombinant polynucleotide that encodes a chimeric receptor. The chimeric receptor enables the lymphocytes, to proliferate in response to an alternative cytokine, preferably a cytokine that is expressed in the lymphocyte at elevated levels in  
25 response to cognate antigen stimulation, or a cytokine that could be administered with reduced toxicity relative to, e.g., IL-2. The chimeric receptor is a fusion of an extracellular region of one receptor (receptor A-R), via a transmembrane region, to an intracellular region of a second receptor (receptor B-R). As discussed below, the receptor may comprise one or more peptide chains. Binding of cognate cytokine to the extracellular region (derived from receptor A-R)  
30 delivers the signal normally associated with receptor B-R. Thus, for example, an activated CTL containing a chimeric GM-CSF:IL-2 receptor, as a result of the CTL production of GM-CSF would proliferate (in response to GM-CSF) in the presence of quantities of IL-2 that would normally be limiting. Cytoplasmic domains of suitable receptors are chosen dependent upon the type of lymphocyte to be stimulated. For example, if the lymphocyte is a CTL,  
35 suitable cytoplasmic regions may be obtained from a receptor for a cytokine the lack of which



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would limit proliferation in the absence of cytokines produced by T<sub>H</sub>-cells, for example, IL-2. Suitable receptor regions for the extracellular domains can be derived from a receptor recognizing a cytokine that is to be present in non-limiting quantities. For example, for CTLs, the receptors include those that bind granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, gamma-interferon (IFN- $\gamma$ ) and tumor necrosis factor-beta (TNF- $\beta$ ). As is well known in the art, suitable transmembrane domains will exhibit characteristics such as hydrophobicity that promote their stable incorporation into the cellular membrane. Logically, a convenient source of such a transmembrane domain will be the transmembrane domains of receptors A-R or B-R. Preferably, therefore, the transmembrane domain is derived from cytokine receptor A-R or, more preferably, from receptor B-R. However, a large variety of other transmembrane domains have been described in the art and these can also be used in the invention.

A prototypical chimeric receptor is shown in Figure 1. The receptor consists of the extracellular region of a heterologous cytokine receptor, in the figure the GM-CSF receptor (GM-CSF-R), fused to the transmembrane and cytoplasmic regions of a cytokine receptor recognizing a cytokine that in the absence of T<sub>H</sub>-cells is normally limiting, in the figure IL-2R. This receptor should bind the heterologous or alternative cytokine (in the figure GM-CSF) extracellularly but deliver the proliferative signal of the cytokine receptor whose cytoplasmic domain is used as part of the chimeric construct. In the construct of Figure 1, it would be that of the IL-2R. The chimeric receptors may be expressed in the lymphocytes under the control of a constitutive promoter (PRcon), and therefore, always be present on the cell surface. Proliferation of the cells occurs when the cytokine recognized by the extracellular domain binds to the chimeric receptor. If the cytokine is made by the activated lymphocyte, activation creates an autocrine growth loop. In the alternative, it may be desirable to include in the chimeric receptor extracellular domain a receptor fragment that recognizes a cytokine that may be furnished as part of a therapeutic regimen. It may alternatively be desirable to use a promoter that is subject to induction or repression. A variety of such promoters are known in the art.

"Lymphocytes" as used herein, are spherical cells with a large round nucleus (which may be indented) and scanty cytoplasm. They are cells that specifically recognize and respond to non-self antigens, and are responsible for development of specific immunity. Included within "lymphocytes" are B-lymphocytes and T-lymphocytes of various classes.

"Cytotoxic T lymphocytes" or "CTLs" are T cells which bear the CD3 cell surface determinant and mediate the lysis of target cells bearing cognate antigens. CTLs may be of either the CD8<sup>+</sup> or CD4<sup>+</sup> phenotype. CTLs are generally antigen-specific and MHC-restricted





in that they recognize antigenic peptides only in association with the Major Histocompatibility Complex (MHC) molecules on the surface of target cells. CTLs may be specific for a wide range of viral, tumor or allospecific antigens, including HIV, EBV, CMV and a wide range of tumor antigens. Some CTLs, however, may not be antigen specific, for example, some cloned  
5 CTLs can be induced to lose some of their specificity for their cognate antigen by culture in abnormally high concentrations of IL-2 (Brooks et al., Immunol. Rev. 72:43; 1983).

A " $T_H$ -independent" CTL is, relative to the CTL from which it was derived, capable of enhanced growth or proliferation in the presence of limiting quantities of  $CD4^+$  T helper ( $T_H$ ) cells and/or a cytokine normally required for proliferation or growth. Growth or proliferation  
10 may be measured, for example, by any in vitro proliferation or growth assay or by any assay measuring the ability of the CTL to persist in vivo. Specific examples of suitable assays are disclosed infra. CTLs capable of enhanced growth or viability may have augmented ability to destroy target cells bearing the foreign antigens or provide long-term immunologic memory.

"Cytokine" refers to a polypeptide that is a soluble intercellular signalling molecule,  
15 including for example, the interleukins, interferons, colony stimulating factors and TNFs.

The term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations  
20 and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as the modifications known in the art, both naturally occurring and non-naturally occurring.

"Transformation", as used herein, refers to the insertion of an exogenous  
25 polynucleotide into a host cell, irrespective of the method used for the insertion, for example, direct uptake, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host cell genome.

"Treatment" as used herein refers to prophylaxis and/or therapy.

30 "Helper T cells" or "helper cells" or " $T_H$ -cells" are a functional subclass of T cells which can help to generate cytotoxic T cells and cooperate with B cells in the production of an antibody response. Helper cells usually recognize antigen in association with class II MHC molecules.



An "antigen specific T cell clone" is comprised of the progeny of a single cell; the cells in this type of clone are of the same phenotype and are all targeted towards the same antigen. Methods of preparing antigen-specific T cell clones are known in the art.

The term "recombinant expression vector" refers to a replicable unit of DNA or RNA  
5 in a form which is capable of being introduced into a target cell by transformation, electroporation, transduction or viral infection, and which codes for the expression of a heterologous structural coding sequence, for example, a cytokine, which is transcribed into mRNA and translated into protein under the control of elements having a regulatory role in gene expression. Such vectors will preferably also contain appropriate transcription and  
10 translation initiation and termination sequences.

"Recombinant," as used herein, means that a particular DNA sequence is the product of various combinations of cloning, restriction, and ligation steps resulting in a construct having a structural coding sequence distinguishable from homologous sequences found in natural systems. Generally, DNA sequences encoding the structural coding sequence, for example  
15 cytokines, can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being expressed in a recombinant transcriptional unit. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. Genomic DNA containing the relevant sequences could  
20 also be used. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where such sequences do not interfere with manipulation or expression of the coding regions.

"Recombinant host cells", "host cells", "cells", "cell lines", "cell cultures", and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular  
25 entities refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer polymucleotides, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

30 A CTL is "cytolytically specific for" cells expressing tumor or viral antigens if the CTL is capable of selectively recognizing and lysing the cells bearing the tumor or viral antigen. A CTL is "cytolytically reactive against" cells expressing tumor or viral antigens if the CTL is capable of lysing the cells bearing the tumor or viral antigen, without regard to its ability to selectively recognize such cells.



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"Antigen specific expression" refers to expression that occurs when the T cell recognizes its cognate antigen.

"Cognate antigen" refers to antigen, a peptide of which is associated with an MHC molecule, such that it forms a ligand that binds to a lymphocyte that recognizes it and causes triggering of signals for the effector function of the cell and/or for proliferation.

An "activated lymphocyte" is one that as a result of binding of a cognate antigen is producing polypeptide factors (including, for example, cytokines) at a level that is elevated relative to the lymphocyte without the bound cognate antigen.

A "transcriptional regulatory region" encompasses all the elements necessary for transcription, and may include elements necessary for regulation. Thus, a transcriptional regulatory region includes at least the promoter sequence, and may also include other regulatory sequences such as enhancers, and transcription factor binding sites.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For instance, a promoter is operably linked to a coding sequence if the promoter affects its transcription or expression.

The term "recombinant" polynucleotide or nucleic acid refers to one which is not naturally occurring, or is made by the artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Such is usually done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a desired combination of functions.

The "sense strand" of a nucleic acid contains the sequence that has sequence homology to that of the cognate mRNA. The "anti-sense strand" contains a sequence which is complementary to that of the "sense strand".

An "individual" as used herein refers to vertebrates, particularly members of the mammalian species, and includes but is not limited to domestic animals, sports animals, and primates, including humans.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Sambrook, Fritsch, and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition (1989), OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait Ed., 1984), ANIMAL CELL CULTURE (R.I. Freshney, Ed., 1987), the series



METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.M. Miller and M.P. Calos eds. 1987), HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, (D.M. Weir and C.C. Blackwell, Eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Siedman, J.A. Smith, and K. Struhl, eds., 1987), and CURRENT PROTOCOLS IN IMMUNOLOGY (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach and W. Strober, eds., 1991). All patents, patent applications, and publications mentioned herein, both supra and infra, are hereby incorporated herein by reference.

The design of some of the chimeric receptors of the invention is as follows, and uses the IL-2R as an illustration. The IL-2R, including human IL-2R, is comprised of three chains, two of which ( $\beta$  and  $\gamma$ ) are required for signal transduction. (See Hatakeyama et al. (1989), *Science* 244:379-382; and Takeshita et al. (1992), *Science* 257:551-556, for the coding sequences and description of functionality of the IL-2R  $\beta$  and  $\gamma$  chains; see also, Nelson, B.H. et al. (1994) *Nature* (in press)). Thus, a given chimeric receptor is comprised of two chains containing three regions or domains: cytoplasmic, transmembrane, and extracellular. As will be apparent, the transmembrane domain may conveniently be derived from the same source as the cytoplasmic or the extracellular domain, but transmembrane domains from other sources can likewise be used. In an example based on IL-2, one chain of the chimeric receptor has an IL-2R  $\beta$  chain transmembrane region and cytoplasmic region while the other chain has a  $\gamma$  chain transmembrane region and cytoplasmic region. The number of chain types in the extracellular region of the chimeric receptor depends on the heterologous cytokine receptor selected to contribute to this domain. For example, hetero-oligomeric type cytokine receptors (for example, IL-2R, IL-3R, IL-5R, IFN- $\gamma$ -R, GM-CSF-R and IL-6-R) require at least two distinct subunit types to form a high affinity receptor. The IL-4 and IL-7 receptors are also hetero-oligomeric type receptors in that they have been reported to utilize a second ( $\gamma$ ) chain for signalling, which  $\gamma$  chain appears to be the same as that of the IL-2 receptor. (See, e.g., Kondo, M., et al. (1993), *Science* 262:1874-1877). Other types of cytokine receptors have high affinity ligand binding by itself (i.e. monomeric) or form a high affinity receptor as a homodimer or homo-oligomer (e.g., c-kit-R, TNF-R, Epo-R and G-CSF-R). A discussion of the properties and coding sequences of a number of cytokine receptors is found in a review by Miyajima et al. (1992), *Ann. Rev. Immunol.* 10:295:331.

Figure 2 illustrates the design of a prototypic chimeric receptor in which the non-IL-2R extracellular domain is from a cytokine receptor that contains a single type chain, for example, c-kit. The figure illustrates the normal c-kit on the left and the normal IL-2R  $\beta$  and  $\gamma$  chains on the right. The middle of the drawing shows a two-chain chimeric receptor containing the





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extracellular region of c-kit fused to the transmembrane and cytoplasmic region of the IL-2R  $\beta$  chain ( $\kappa:\beta$ ) and of the  $\gamma$  chain ( $\kappa:\gamma$ ). Other examples of this type of construct can be formed using extracellular domains from other one chain type receptors, e.g., TNF-R and G-CSF-R.

An illustration of another type of prototypic chimeric receptor is shown in the inset of Figure 1. In this illustration the non-IL-2R extracellular domain is from a cytokine receptor that contains two types of chains, for example, GM-CSF-R. In the figure, the extracellular region of the  $\alpha$ -subunit of GM-CSF-R is fused to the transmembrane region and cytoplasmic region of the  $\gamma$  chain of IL-2R; the extracellular region of the  $\beta$ -subunit of GM-CSF-R is fused to the transmembrane region and cytoplasmic region of the  $\beta$  chain of IL-2R. See also, the illustration in Figure 5.

Preferred examples of chimeric receptors contain the cytoplasmic and transmembrane regions of the human IL-2R  $\beta$  and  $\gamma$  chains and extracellular domains derived from the following receptors: human and murine GM-CSF-R; human and murine IFN $\gamma$ -R; human and murine IL-3R; human and murine G-CSF-R; human and murine IL-4R; human and murine IL-7R; human and murine c-kit-R; and human and murine Epo-R.

The chimeric receptors may be constructed from cDNAs encoding the desired segments, although other methods are readily apparent to those of ordinary skill in the art. In one method, for example, the chimeric receptor DNA is prepared by providing cloned cDNAs encoding the upstream extracellular region from a heterologous cytokine receptor and the downstream IL-2R transmembrane and cytoplasmic domains. These cloned cDNAs, if prepared by restriction enzyme digestion, may contain unwanted sequences that would intervene in the fusion. The unwanted sequences are removable by techniques known to those of ordinary skill in the art, including loop-out site-directed mutagenesis or splice-overlap extension polymerase chain reaction (PCR). The sequence of the chimeric cDNA encoding the receptor may then be confirmed by standard DNA sequencing methods. Specific examples of such chimeric receptors are illustrated in more detail below.

The polynucleotide region encoding the chimeric receptors are generally operably linked to control regions that allow expression of the chimeric receptor in a host cell, particularly a CTL. Control regions include, at least, a promoter and a ribosomal binding site, and may also include, inter alia, enhancer regions, splice regions, polyadenylation regions, transcription and/or translation termination regions, and transcription and/or translation factor binding sites. These control regions may be present in recombinant vectors, particularly in recombinant expression vectors.

The ability of the chimeric receptor to support proliferation of the activated CTL is readily demonstrated by techniques known in the art. For example, activated cell lines that



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express the chimeric receptors can be tested for growth in the absence of the cytokine whose signal is normally associated with the cytoplasmic domain portion of the receptor, but in the presence of the cytokine recognized by the extracellular domain cytokine receptor portion of the chimeric construct.

5           The invention contemplates transforming lymphocytes with at least one type of chimeric receptor to lessen the dependence upon at least one cytokine. However, it is also within the invention to transform lymphocytes with multiple forms of chimeric receptors that may, for example, regulate the lessened dependency on a cytokine, and/or lessen and/or regulate the dependency on two or more cytokines.

10           Techniques for nucleic acid manipulation are described generally, for example, in Sambrook et al. (1989), supra., Ausubel et al. (1987), supra. and in Annual Reviews of Biochemistry (1992) 61:131-156. Reagents useful in applying such techniques, such as restriction enzymes and the like, are widely known in the art and commercially available from a number of vendors.

15           Large amounts of the polynucleotides used to create the cells of the present invention may be produced by replication in a suitable host cell. The natural or synthetic polynucleotide fragments coding for a desired fragment may be incorporated into recombinant nucleic acid constructs, typically polynucleotide constructs, capable of introduction into and replication in a prokaryotic or eukaryotic cell. Usually the constructs will be suitable for replication in a  
20           unicellular host, such as yeast or bacteria, but may also be intended for introduction to, with and without and integration within the genome, cultured mammalian or plant or other eukaryotic cell lines. Purification of nucleic acids produced by the methods of the present invention can be achieved by methods known in the art and described, e.g., in Sambrook et al. (1989) and Ausubel et al. (1987).

25           The polynucleotides used in the present invention may also be produced in part or in total by chemical synthesis, e.g., by the phosphoramidite method described by Beaucage and Carruthers (1981) *Tetra. Letts.* 22:1859-1862 or the triester method according to Matteucci et al. (1981) *J. Am. Chem. Soc.* 103:3185, and may be performed on commercial automated oligonucleotide synthesizers. A double-stranded fragment may be obtained from the single  
30           stranded product of chemical synthesis either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Polynucleotide constructs prepared for introduction into a prokaryotic or eukaryotic host cell for replication will typically comprise a replication system recognized by the host,



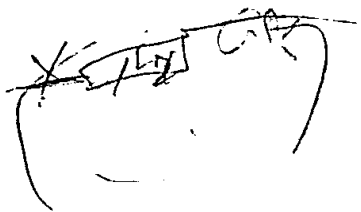
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including the intended recombinant polynucleotide fragment encoding the desired polypeptide. Such vectors may be prepared by means of standard recombinant techniques well known in the art and discussed, for example, in Sambrook *et al.* (1989) or Ausubel *et al.*

Preferably, the polynucleotide construct will contain a selectable marker, a gene  
5 ~~encoding a protein necessary for the survival or growth of a host cell transformed with the~~  
~~vector. The presence of this gene ensures the growth of only those host cells which express the~~  
~~inserts. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other~~  
~~toxic substances, e.g. ampicillin, neomycin, methotrexate, etc.; (b) complement auxotrophic~~  
~~deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene~~  
10 ~~encoding D-alanine racemase for *Bacilli*. The choice of the proper selectable marker will~~  
~~depend on the host cell, and appropriate markers for different hosts are well known in the art.~~

The polynucleotides conferring  $T_H$ -independence upon CTLs (i.e., a lowered  
dependence upon  $T_H$ -cells or one or more cytokines furnished by  $T_H$  cells) may be introduced  
into the desired type Ag-specific T cell by means known in the art, including, for example,  
15 transformation, electroporation, lipofection, and transduction, including the use of  
adenoassociated viral (AAV) vectors, and particularly using methods of retroviral gene transfer  
known in the art.

Various infection techniques have been developed which utilize recombinant infectious  
virus particles for gene delivery. This represents a preferred approach to the present invention.  
20 The viral vectors which have been used in this way include virus vectors derived from simian  
virus 40 (SV40; Karlsson *et al.*, Proc. Natl. Acad. Sci. USA 84 82:158, 1985), adenoviruses  
(Karlsson *et al.*, EMBO J. 5:2377, 1986), adeno-associated virus (AAV) (B.J. Carter, Current  
Opinion in Biotechnology 1992, 3:533-539), and retroviruses (Coffin, 1985, pp. 17-71 in Weiss  
*et al.* (eds.), RNA Tumor Viruses, 2nd ed., Vol. 2, Cold Spring Harbor Laboratory, New  
25 York). Thus, gene transfer and expression methods are numerous but essentially function to  
introduce and express genetic material in mammalian cells. Several of the above techniques  
have been used to transduce hematopoietic or lymphoid cells, including calcium phosphate  
transfection (Berman *et al.*, *supra*, 1984), protoplast fusion (Deans *et al.*, *supra*, 1984),  
electroporation (Cann *et al.*, Oncogene 3:123, 1988), and infection with recombinant  
30 adenovirus (Karlsson *et al.*, *supra*; Reuther *et al.*, Mol. Cell. Biol. 6:123, 1986), adeno-  
associated virus (LaFace *et al.*, *supra*) and retrovirus vectors (Overell *et al.*, Oncogene 4:1425,  
1989). Primary T lymphocytes have been successfully transduced by electroporation (Cann *et al.*,  
*supra*, 1988) and by retroviral infection (Nishihara *et al.*, Cancer Res. 48:4730, 1988;  
Kasid *et al.*, *supra*, 1990; and Riddell, S. *et al.*, Human Gene Therapy 3:319-338, 1992).





5 Retroviral vectors provide a highly efficient method for gene transfer into eukaryotic cells and is the preferred method for the delivery of the polynucleotides of the invention into the T<sub>H</sub>-dependent CTLs. Moreover, retroviral integration takes place in a controlled fashion and results in the stable integration of one or a few copies of the new genetic information per cell.

Retroviruses are a class of viruses which replicate using a virus-encoded, RNA-directed DNA polymerase, or reverse transcriptase, to replicate a viral RNA genome to provide a double-stranded DNA intermediate which is incorporated into chromosomal DNA of an avian or mammalian host cell. Most retroviral vectors are derived from murine retroviruses.

10 Retroviruses adaptable for use in accordance with the present invention can, however, be derived from any avian or mammalian cell source. These retroviruses are preferably amphotropic, meaning that they are capable of infecting host cells of several species, including humans. A characteristic feature of retroviral genomes (and retroviral vectors used as described herein) is the retroviral long terminal repeat, or LTR, which is an untranslated region  
15 of about 600 base pairs found in slightly variant forms at the 5' and 3' ends of the retroviral genome. When incorporated into DNA as a provirus, the retroviral LTR includes a short direct repeat sequence at each end and signals for initiation of transcription by RNA polymerase II and 3' cleavage and polyadenylation of RNA transcripts. The LTR contains all other *cis*-acting sequences necessary for viral replication.

20 A "provirus" refers to the DNA reverse transcript of a retrovirus which is stably integrated into chromosomal DNA in a suitable host cell, or a cloned copy thereof, or a cloned copy of unintegrated intermediate forms of retroviral DNA. Forward transcription of the provirus and assembly into infectious virus occurs in the presence of an appropriate helper virus or in a cell line containing appropriate sequences enabling encapsidation without  
25 coincident production of a contaminating helper virus. Mann et al. (Cell 33:153, 1983) describe the development of cell lines (e.g., Ψ2) which can be used to produce helper-free stocks of recombinant retrovirus. These cells lines contain integrated retroviral genomes which lack sequences required *in cis* for encapsidation, but which provide all necessary gene product *in trans* to produce intact virions. The RNA transcribed from the integrated mutant provirus  
30 cannot itself be packaged, but these cells can encapsidate RNA transcribed from a recombinant retrovirus introduced into the same cell. The resulting virus particles are infectious, but replication-defective, rendering them useful vectors which are unable to produce infectious virus following introduction into a cell lacking the complementary genetic information enabling encapsidation. Encapsidation in a cell line harboring *trans*-acting elements encoding an





ecotropic viral envelope (e.g.,  $\Psi$ 2) provides ecotropic (limited host range) progeny virus. Alternatively, assembly in a cell line containing amphotropic packaging genes (e.g., PA317, ATCC CRL 9078; Miller and Buttimore, Mol. Cell. Biol. 6:2895, 1986) provides amphotropic (broad host range) progeny virus. Such packaging cell lines provide the necessary retroviral gag, pol and env proteins *in trans*. This strategy results in the production of retroviral particles which are highly infectious for mammalian cells, while being incapable of further replication after they have integrated into the genome of the target cell. The product of the *env* gene is responsible for the binding of the retrovirus to viral receptors on the surface of the target cell and therefore determines the host range of the retrovirus. The PA 317 cells produce retroviral particles with an amphotropic envelope protein, which can transduce cells of human and other species origin. Other packaging cell lines produce particles with ecotropic envelope proteins, which are able to transduce only mouse and rat cells.

Numerous retroviral vector constructs have been used successfully to express many foreign genes (see, e.g., Coffin, in Weiss et al. (eds.), RNA Tumor Viruses, 2nd ed., vol. 2 (Cold Spring Harbor Laboratory, New York, 1985, pp. 17-71). Retroviral vectors with inserted sequences are generally functional, and few sequences that are consistently inhibitory for retroviral infection have been identified. Functional polyadenylation motifs inhibit retroviral replication by blocking retroviral RNA synthesis, and there is an upper size limit of approximately 11 kb of sequence which can be packaged into retroviral particles (Coffin, supra, 1985); however, the presence of multiple internal promoters, initially thought to be problematic (Coffin, supra, 1985), was found to be well tolerated in several retroviral constructs (Overell et al., Mol. Cell. Biol. 8:1803, 1983).

Retroviral vectors have been used as genetic tags by several groups to follow the development of murine hematopoietic stem cells which have been transduced *in vitro* with retrovirus vectors and transplanted into recipient mice (Williams et al., Nature 310:476, 1984; Dick et al., Cell 42:71, 1985; Keller et al., Nature 318:149, 1985). These studies have demonstrated that the infected hematopoietic cells reconstitute the hematopoietic and lymphoid tissue of the recipient animals and that the cells display a normal developmental potential *in vivo*. The marked cells can be visualized using any of a number of molecular biological techniques which can demonstrate the presence of the retroviral vector sequences, most notably Southern analysis and PCR (polymerase chain reaction). The ability to mark cells genetically using retroviral vectors is also useful in clinical settings in which the technique can be used to track grafts of autologous cells. This approach has already been used to track TILs (tumor-infiltrating lymphocytes) in patients given TIL therapy for terminal cancer treatment by



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Rosenberg et al. (N. Engl. J. Med. 323:570, 1990). The transduction of these cells with the marker gene was not associated with in vitro cellular dysfunction (Kasid et al., Proc. Natl. Acad. Sci. USA 87:473, 1990).

5 Many gene products have been expressed in retroviral vectors. This can either be achieved by placing the sequences to be expressed under the transcriptional control of the promoter incorporated in the retroviral LTR, or by placing them under the control of a heterologous promoter inserted between the LTRs. The latter strategy provides a way of coexpressing a dominant selectable marker gene in the vector, thus allowing selection of cells which are expressing specific vector sequences.

10 It is contemplated that overexpression of a stimulatory factor (for example, a lymphokine or a cytokine) may be toxic to the treated individual. Therefore, it is within the scope of the invention to include gene segments that cause the T cell clones of the invention to be susceptible to negative selection in vivo. By "negative selection" is meant that the infused cell can be eliminated as a result of a change in the in vivo condition of the individual. The negative selectable phenotype may result from the insertion of a gene that confers sensitivity to  
15 an administered agent, for example, a compound. Negative selectable genes are known in the art, and include, *inter alia* the following: the *Herpes simplex* virus type I thymidine kinase (HSV-I TK) gene (Wigler et al., *Cell* 11:223, 1977) which confers ganciclovir sensitivity; the cellular hypoxanthine phosphoribosyltransferase (HPRT) gene, the cellular adenine  
20 phosphoribosyltransferase (APRT) gene, bacterial cytosine deaminase, (Mullen et al., *Proc. Natl. Acad. Sci. USA* 89:33 (1992)).

In addition, it is useful to include in the T cells a positive marker that enables the selection of cells of the negative selectable phenotype in vitro. The positive selectable marker may be a gene which, upon being introduced into the host cell expresses a dominant phenotype  
25 permitting positive selection of cells carrying the gene. Genes of this type are known in the art, and include, *inter alia*, hygromycin-B phosphotransferase gene (hph) which confers resistance to hygromycin B, the aminoglycoside phosphotransferase gene (*neo* or *aph*) from Tn5 which codes for resistance to the antibiotic G418, the dihydrofolate reductase (DHFR) gene, the adenosine deaminase gene (ADA), and the multi-drug resistance (MDR) gene.

30 Preferably, the positive selectable marker and the negative selectable element are linked such that loss of the negative selectable element necessarily also is accompanied by loss of the positive selectable marker. Even more preferably, the positive and negative selectable markers are fused so that loss of one obligatorily leads to loss of the other. An example of a fused polynucleotide that yields as an expression product a polypeptide that confers both the desired



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positive and negative selection features described above is a hygromycin phosphotransferase thymidine kinase fusion gene (HyTK). Expression of this gene yields a polypeptide that confers hygromycin B resistance for positive selection in vitro, and ganciclovir sensitivity for negative selection in vivo. See Lupton S.D., *et al.*, *Mol. and Cell. Biology* 11:3374-3378, 5 1991. In addition, in preferred embodiments, the polynucleotides of the invention encoding the chimeric receptors are in retroviral vectors containing the fused gene, particularly those that confer hygromycin B resistance for positive selection in vitro, and ganciclovir sensitivity for negative selection in vivo, for example the HyTK retroviral vector described in Lupton, S.D. *et al.* (1991), *supra*. See, also, the description of Bifunctional Selectable Fusion Genes by 10 Lupton, S.D., WO 92/08796 (international publication date 29 May 1992).

The lymphocyte clones of the invention may be used to confer immunity to individuals. By "immunity" is meant a lessening of one or more physical symptoms associated with a response to infection by a pathogen, or to a tumor, to which the lymphocyte response is directed. The amount of cells administered is usually in the range present in normal individuals 15 with immunity to the pathogen. Thus, CD8+ CD4- cells are usually administered by infusion, with each infusion in a range of at least  $10^6$  to  $10^{10}$  cells/m<sup>2</sup>, preferably in the range of at least  $10^7$  to  $10^9$  cells/m<sup>2</sup>. The clones may be administered by a single infusion, or by multiple infusions over a range of time. However, since different individuals are expected to vary in responsiveness, the type and amount of cells infused, as well as the number of infusions and the 20 time range over which multiple infusions are given are determined by the attending physician or veterinarian, and can be determined by routine examination.

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way. 25

#### Example 1

##### Construction of c-kit:IL-2R $\beta$

An XhoI-BamHI fragment of human IL-2R $\beta$  cDNA encompassing most of the extracellular coding region and the entire transmembrane and cytoplasmic regions was cloned 30 into the XhoI and BamHI sites of a modified version of the plasmid pBluescript SK- (Stratagene) in which the NotI-SacI region had been cleaved out, blunt-ended and religated. (Human IL-2R $\beta$  cDNA (see, e.g., Hatakeyama, M., *et al.* (1989), *Science* 244:551-556) was provided by Tada Taniguchi, Osaka, Japan.) This plasmid was then digested with KpnI and HincII, and the 199 bp fragment was replaced with a 1995 bp KpnI-blunted ApaI fragment of 35 murine c-kit cDNA encompassing the entire extracellular and transmembrane coding regions



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and a portion of the cytoplasmic coding region. (Murine c-kit cDNA (see, e.g., Qiu, F. et al. (1988) EMBO J. 7:1003-1011) was provided by Stewart Lyman, Immunex Corp., Seattle, Washington.) The resultant plasmid, pBK $\beta$ , thus had most of the c-kit cDNA upstream of most of the IL-2R $\beta$  cDNA. The fusion was completed by site-directed looping-out mutagenesis. A

5 uracil-containing single-stranded form of this plasmid was prepared using a Muta-Gene Phagemid In Vitro Mutagenesis kit (BioRad) according to the manufacturer's instructions. This was annealed to a fusion primer with the sequence: 5' GCC GAG CCA CGG AAT GTG GGC CTG GAT TTG 3'. Second strand synthesis, ligation, and transformation of *E. coli* with the

10 annealed product was carried out according to the manufacturer's instructions. The resulting plasmid in which the unwanted intervening cDNA sequences had been successfully removed was digested with HincII and SacI and the 859 bp fragment encompassing the fusion site was cloned back into the parent plasmid, pBK $\beta$  which had been similarly digested. This last step minimized the amount of cDNA sequence that had been through the mutagenesis steps, which are error-prone. The sequence of the final plasmid, pBk $\beta$ -EX, was confirmed by standard

15 DNA sequencing methods.

A 2524 bp KpnI (blunted)-BamHI (partial digest) fragment from pBk $\beta$ -EX that encompassed the entire coding region of the k: $\beta$  chimeric cDNA was cloned into the expression vector pH $\beta$ APr-1-neo (Gunning, P. et al. (1987), P.N.A.S. 84:4831-4835) which had been digested with SalI (blunted) and BamHI. This plasmid, called pNk $\beta$ , was then transfected into

20 cells.

## Example 2

### Construction of c-kit:IL-2R $\gamma$

A fragment of human IL-2R $\gamma$  cDNA (Takeshita, T. et al. (1992), Science 257:379-382)

25 was PCR amplified from second strand cDNA derived from the human T cell clone 2D5 using the primers 5' CCGTTGAC CCC ACT CTG TGG AAG TGC 3' and 5' CCGGATCC GGC TAC AGG ACC CTG GGG 3' (the underlined bases encode restriction enzyme sites). The product was then cloned into the HincII and BamHI sites of the modified pBluescript SK-plasmid described above and was subjected to DNA sequencing. This plasmid was then

30 digested with KpnI and HincII, and the 199 bp fragment was replaced with a 1995 bp KpnI-blunted ApaI fragment of murine c-kit cDNA encompassing the entire extracellular and transmembrane coding regions and a portion of the cytoplasmic coding region. The resultant plasmid, pBk $\gamma$ , thus had most of the c-kit cDNA upstream of most of the IL-2R $\gamma$  cDNA. The fusion was completed by PCR splice-overlap extension. The portion of the plasmid encoding

35 the c-kit extracellular region was amplified by PCR using primers A (CCG AGC ACC AGC

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AGT GG) and B (TGC AAA CAG GAA AGG GTG GGC CTG GAT TTG). The underlined region of primer B is actually complementary to the transmembrane region of IL-2R $\gamma$ . The IL-2R $\gamma$  transmembrane and cytoplasmic coding regions were amplified from pBk $\gamma$  by PCR using primers C (CAA ATC CAG GCC CAC CCT TTC CTG TTT GCA) and D (CCGGATCC GGG GTT CAG GTT TCA GGC). Note that primer C is entirely complementary to primer B, and together they encode the desired fusion site. The products of these two PCR reactions were then combined in a single tube where they served as substrates in a third PCR reaction using primers A and D. The complementarity of primers B and C allows the products of the first two PCR reactions to anneal to each other and generate an overlap-extended template. The product of the third PCR reaction thus encoded a chimeric cDNA with the desired fusion site and extending from the annealing site of primer A to that of primer D. This product was then digested with BstX1 and Pst1 to generate an 879 bp fragment that was cloned back into pBk $\gamma$ , which had been similarly digested. The sequence of the final plasmid, pBk $\gamma$ -EX, was confirmed by standard DNA sequencing methods.

15 A 1977 bp Kpn1 (blunted)-BamH1 (partial digest) fragment from pBk $\gamma$ -EX that encompassed the entire coding region of the k $\gamma$  chimeric cDNA was cloned into the expression vector pH $\beta$ APr-1-hygro which had been digested with Sal1 (blunted) and BamH1 (the expression vector pH $\beta$ APr-1-hygro contains the hygromycin sequence derived from RSV.5 (Long, E.O. et al. (1991) Human Immunology 31:229-235) cloned into the expression vector pH $\beta$ APr-1-neo, described above). The resulting plasmid, called pHk $\gamma$ , was then transfected into cells.

### Example 3

#### Construction of pNkit

25 The cDNA encoding wild type murine c-kit was cloned into the expression vector pH $\beta$ aPr-1-neo by ligating a blunt-ended 2970 bp Kpn1-Not1 fragment containing the entire c-kit coding region into pH $\beta$ aPr-1-neo which had been digested with HindIII, blunt-ended and dephosphorylated.

### Example 4

#### Modification of cytokine dependence in a cytotoxic T lymphocyte cell line

#### Expression in T cells

T cells are transfected with plasmids encoding the chimeric receptor, and drug-resistant cell lines selected. To test for expression of the chimeric receptors, drug-resistant cell lines are



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stained with antibody that recognizes the extracellular region and are scanned by flow cytometry. Expression can also be detected by Western blotting.

- Cell lines that express the chimeric receptors are tested for growth in response to the cognate alternative cytokine (i.e., the one recognized by the extracellular domain of the chimeric receptor) in the presence of limiting amounts of the cytokine normally required for growth and/or proliferation, e.g., IL-2.

#### T cell transfections

- Plasmids were linearized by digestion with EcoR1 (for pNk $\beta$  and pNkit) or Xmn1 (for pHk $\gamma$ ) and then purified by phenol-chloroform extraction followed by ethanol precipitation. They were resuspended in distilled water to a concentration of 1 microgram per microliter.

- 10 million CTLL2 cells were washed once with PBS and then resuspended in 0.8 ml PBS. Twenty-five micrograms of linearized plasmid was added, and the cells left at room temperature for 10 minutes. Doubly transfected cells (i.e. pNk $\beta$  and pHk $\gamma$ ) received 25 micrograms of each plasmid. The cells were then placed in a cuvette and electroporated at 250V.1080  $\mu$ F for one second at room temperature. The cells were then placed in complete media (Click's + 10% FCS, 1% glutamine, 1% pen-strep) at 37°C for 16-20 hours. At this time, the cells were spun down, resuspended in fresh media and incubated at 37°C for 24 additional hours. Forty-eight hours post-transfection, cells were selected by the addition of neomycin (1 mg/ml) for groups pNkit, pNk $\beta$  + pHk $\gamma$  or hygromycin B (0.5 mg/ml) for the pHk $\gamma$  group. Drug-resistant cell lines typically grew out in one to two weeks. In the case of the pNk $\beta$  + pHk $\gamma$  cells, the neomycin resistant line was then further selected by removing IL-2 and adding recombinant murine stem cell factor (200 ng/ml) to the cultures.

#### Flow cytometry:

- 500,000 to one million cells were washed twice in staining solution (Hank's media containing 2% fetal calf serum) and resuspended in 0.1 ml staining solution. One microgram of the monoclonal antibody ACK-2 (Gibco/BRL) which recognizes the extracellular region of murine c-kit was added and the cells were left on ice for 30 minutes. After one wash with staining solution, the cells were resuspended in 0.1 ml staining solution containing a fluorescein-conjugated goat anti-rat secondary antibody (Tago Immunochemicals) and left on ice for thirty minutes. After one wash in staining solution the cells were resuspended in 0.25 ml 0.5% paraformaldehyde.

- The fluorescent intensity of the cells was measured using a Becton-Dickinson FACS Scanner.



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Proliferation assay:

CTLL2 lines were washed twice in RPMI and resuspended in complete medium (Click's + 10% FCS, 1% glutamine, 1% pen-strep) at a concentration of 20,000 per ml. Cells were plated in flat-bottom 96-well plates, 0.2 ml per well, with either no added cytokines, or recombinant human IL-2 (100 units./ml), or recombinant murine stem cell factor (200 ng/ml) and incubated at 37°C for 20 hours. Then 2.5 microcuries of tritiated thymidine was added and the incubation continued for an additional 4 hours. Cells were harvested, placed in scintillation fluid, and incorporated thymidine was measured using a scintillation counter.

10 Results

The results provide evidence that the c-kit:IL-2 chimeric receptor is functional. Figure 3 shows a flow cytometric analysis of the expression of the chimeric receptors in CTLL2, a mouse T cell line that is highly IL-2 dependent for growth and survival. The cells were stained with an antibody against c-kit. The parental cell line is negative for c-kit, whereas cells transfected with k:γ alone, k:β alone, and k:γ and k:β together, or normal c-kit alone are all positive.

Figure 4 shows the proliferative response of these cells to murine stem cell factor (SCF), the natural ligand for c-kit. All the cell lines respond to IL-2. Significantly, cells expressing both k:β and k:γ also respond to SCF. Normal CTLL2 cells die within 24 hours of withdrawal from IL-2. However, the k:β/k:γ transfectants have been maintained for more than a year in the presence of SCF and absence of exogenously added IL-2.

Example 5Modification of cytokine dependence25 in a pro-B cell line

Similar results to those described in Example 4, were obtained when k:β/k:γ chimeric receptors were expressed in BAF3 cells (a murine pro-B cell in which growth is normally responsive to IL-3, but responsive to IL-2 if IL-2R is expressed from a transfectant in gene).

Some cell proliferation was also observed in BAF3 cells transfected with k:β chains alone. However, a significant indicator of the generation of a normal IL-2 response is the up-regulation of IL-2Rα (See, e.g., Smith, K.A. et al. (1985) P.N.A.S. 82:864-868). Both CTLL2 and BAF3 cells exhibited up-regulation of IL-2Rα when transfected with a combination of k:β and k:γ chains. Neither CTLL2 nor BAF3 cells exhibited up-regulation of IL-2Rα when transfected with k:β chains alone, nor with k:γ chains alone. These results tend to confirm that, for recreating the normal intracellular response of receptors having hetero-



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oligomeric signalling chains, like the  $\beta$  and  $\gamma$  chains of the IL-2 receptor, it is preferable to incorporate each type of cytoplasmic domain into the chimeric receptors, as described above.

#### Example 6

5                    Modification of cytokine dependence  
                      in a helper T lymphocyte line

Similar results to those described in Example 4, were obtained when the chimeric receptors were expressed in D10.G4.1 cells. The latter cells are from a murine CD4<sup>+</sup> helper T cell clone which normally requires IL-2 for growth. See Kaye et al. (1983), J. Exp. Med.  
10    158:836-856.

#### Example 7

Modification of cytokine dependence  
                      in a murine CD8<sup>+</sup> CTL line

15            Similar results to those described in Example 4, were obtained when the chimeric receptors were expressed in L3 cells. The latter cells are from a murine CD8<sup>+</sup> T cell clone. See Siu, G. et al. (1992), Molec. Cell Biol. 12:1592-1604.

20

The principles and teachings described above have been applied to produce additional embodiments, some of which are described below, further demonstrating the utility of this invention. Included, for example, are additional chimeric receptors demonstrating the usefulness of the invention in T lymphocytes.

25

#### Example 8

##### Construction of GM $\beta$ /2 $\beta$

An XhoI-BamHI fragment of human IL-2R $\beta$  cDNA encoding most of the extracellular coding region and the entire transmembrane and cytoplasmic regions was cloned into the XhoI and BamHI sites of a modified version of the plasmid pBluescript SK- (Stratagene) in which the NotI-SacI region had been cleaved out, blunt-ended and religated. This plasmid was then digested with KpnI and HincII, and ligated to an XhoI/blunted EcoRI fragment of cDNA encoding the entire extracellular and transmembrane coding regions of human GM-CSF-R $\beta$   
30            (KH97) (Hayashida, K. et al. (1990) P.N.A.S. 87:9655-9659). Exact fusions between  
35





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extracellular and transmembrane regions were made by overlap extension PCR using the complementary fusion primers GCCGAGCCACGGAATCGACTCGGTGTCCCA and TGGGACACCGAGTCGATTCCGTGGCTCGGC. The entire coding region of the GM $\beta$ /2 $\beta$  chimeric cDNA was cloned into the expression vector pH $\beta$ APr-1-neo, generating the plasmid pNG $\beta$ . Fusion sites and flanking regions of the GM $\beta$ /2 $\beta$  construct were confirmed by standard DNA sequencing methods.

### Example 9

#### Construction of GM $\alpha$ /2 $\gamma$

A fragment of human IL-2R $\gamma$  cDNA was PCR amplified from second strand cDNA derived from the human T cell clone 2D5 using the primers 5' CCGTTGAC CCC ACT CTG TGG AAG TGC 3' and 5' CCGGATCC GGC TAC AGG ACC CTG GGG 3' (the underlined bases encode restriction enzyme sites.) The product was then cloned into the HincII and BamHI sites of the modified pBluescript SK- plasmid described above and was subjected to DNA sequencing. This plasmid was then digested with XhoI and HincII, and ligated to an XhoI/blunted EcoRI fragment of human GM-CSF-R $\alpha$  cDNA (Gearing, D.P. et al. (1989) EMBO J. 8:3667-3676) encoding the entire extracellular and transmembrane coding regions and a portion of the cytoplasmic coding region. Exact fusions between extracellular and transmembrane regions were made by overlap extension PCR using the complementary fusion primers. TGCAAACAGGAAAGGCCCGTCGTCAGAACC and GGTTCTGACGACGGGCCTTTCCTGTTTGCA. The entire coding region of the GM $\alpha$ /2 $\gamma$  chimeric cDNA was cloned into the expression vector pH $\beta$ APr-1-hygro, generating the plasmid pHG $\gamma$ . Fusion sites and flanking regions of the GM $\alpha$ /2 $\gamma$  construct were confirmed by standard DNA sequencing methods.

### Example 10

#### Modification of cytokine dependence in a cytotoxic T lymphocyte cell line

#### T Cell Transfections

Plasmids were linearized by digestion with EcoRI (for pNG $\beta$ ) or XmnI (for pHG $\gamma$ ) and purified by phenol-chloroform extraction followed by ethanol precipitation. They were resuspended in distilled water to a concentration of 1 microgram per microliter.

10 million CTLL2 cells were washed once with PBS and then resuspended in 0.8ml PBS. Twenty-five micrograms of linearized plasmid was added, and the cells left at room



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temperature for 10 minutes. The cells were then placed in a cuvette and electroporated at 250V, 980  $\mu$ F for one second at room temperature. The cells were then placed in complete media (Click's + 10% FCS, 1% glutamine, 1% pen-strep) at 37°C for 16-20 hours. At this time, the cells were spun down, resuspended in fresh media and incubated at 37°C for 24 additional hours. Forty-eight hours post-transfection, cells were selected by the addition of neomycin (1 mg/ml) for pNG $\beta$  or hygromycin B (0.5 mg/ml) for pHG $\gamma$ . Drug-resistant cell lines typically grew out in one to two weeks. To generate cells expressing both GM $\beta$ /2 $\beta$  and GM $\alpha$ /2 $\gamma$ , the neomycin resistant line expressing GM $\beta$ /2 $\beta$  was electroporated with the plasmid pHG $\gamma$  and then selected for expression of GM $\alpha$ /2 $\gamma$  by removing IL-2 and adding recombinant human GM-CSF (100 ng/ml) to the cultures.

#### Proliferation Assays

CTLL2 lines were washed twice in RPMI and resuspended in complete medium (Click's + 10% FCS, 1% glutamine, 1% pen-strep) at a concentration of 20,000 per ml. Cells were plated in flat-bottom 96-well plates, 0.2 ml per well, with either no added cytokines, or recombinant human IL-2 (5 units/ml), or recombinant human GM-CSF (10 ng/ml) and incubated at 37°C for 20 hours. Then 2.5 microcuries of tritiated thymidine was added and the incubation continued for an additional 4 hours. Cells were harvested, placed in scintillation fluid, and incorporated thymidine was measured using a scintillation counter.

Figure 6 shows the proliferative response of these cells to IL-2 and GM-CSF. All of the cell lines respond to IL-2. Significantly, cells expressing both GM $\beta$ /2 $\beta$  and GM $\alpha$ /2 $\gamma$  exhibit a proliferative response to GM-CSF, further demonstrating the usefulness of the invention.

#### Utility

The polynucleotides of the invention that encode chimeric receptors are useful for the production of lymphocytes that have a lessened requirement for one or more cytokines for proliferation. The cells containing the chimeric receptors with a lessened dependence on one or more cytokines are useful in, inter alia, immunotherapy.



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Claims

1. A chimeric receptor comprising a first peptide chain, said first peptide chain comprising an extracellular domain derived from cytokine receptor A-R that binds cytokine A, joined, via a transmembrane domain, to a cytoplasmic domain derived from cytokine receptor B-R that binds cytokine B, wherein cytokine B is a cytokine that is normally required by a lymphocyte for growth and proliferation and wherein the chimeric receptor expressed in said lymphocyte lessens the growth dependency of the lymphocyte on cytokine B in the presence of cytokine A.
2. The chimeric receptor of claim 1, wherein said cytokine receptor B-R normally comprises two peptide chains that are required for signal transduction, and wherein said chimeric receptor comprises a second peptide chain, said second peptide chain comprising a cytoplasmic domain derived from cytokine receptor B-R.
3. The chimeric receptor of claim 2, wherein said second peptide chain comprises an extracellular domain derived from cytokine receptor A-R that binds cytokine A, joined, via a transmembrane domain, to a cytoplasmic domain derived from cytokine receptor B-R that binds cytokine B.
4. The chimeric receptor of claim 3, wherein cytokine receptor B-R is a hetero-oligomeric type cytokine receptor and wherein the cytoplasmic domain of said second peptide chain is different from the cytoplasmic domain of said first peptide chain.
5. The chimeric receptor of claim 4, wherein cytokine receptor B-R is selected from the group consisting of an interleukin-2 receptor (IL-2R), an interleukin-3 receptor (IL-3R), an interleukin-4 receptor (IL-4R), an interleukin-5 receptor (IL-5R), an interleukin-6 receptor (IL-6R), an interleukin-7 receptor (IL-7R), a  $\gamma$ -interferon receptor (IFN $\gamma$ -R) and a granulocyte/macrophage colony stimulating factor receptor (GM-CSF-R).
6. The chimeric receptor of claim 4, wherein the extracellular domains of the first and second peptide chains are derived from a first hetero-oligomeric type cytokine receptor, and wherein the cytoplasmic domains of the first and second peptide chains are derived from a second hetero-oligomeric type cytokine receptor.



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7. The chimeric receptor of claim 6, wherein the cytoplasmic domains of the first and second peptide chains are derived from an interleukin-2 receptor (IL-2R).

8. The chimeric receptor of claim 7, wherein the extracellular domains of the first and second peptide chains are derived from a cytokine receptor selected from the group consisting of a granulocyte/macrophage colony stimulating factor receptor (GM-CSF-R), a  $\gamma$ -interferon receptor (IFN $\gamma$ -R), an interleukin-3 receptor (IL-3R), an interleukin-4 receptor (IL-4R) and an interleukin-7 receptor (IL-7R).

9. The chimeric receptor of claim 3, wherein cytokine receptor A-R is a cytokine receptor having high affinity ligand binding as a single chain or by homomultimerization.

10. The chimeric receptor of claim 9, wherein cytokine receptor A-R is selected from the group consisting of c-kit receptor (c-kit-R), tumor necrosis factor receptor (TNF-R), erythropoietin receptor (Epo-R) and granulocyte colony stimulating factor receptor (G-CSF-R).

11. The chimeric receptor of claim 3, wherein the extracellular domains of the first and second peptide chains are derived from a cytokine receptor having high affinity ligand binding as a single chain or by homomultimerization, and wherein the cytoplasmic domains of the first and second peptides are derived from a hetero-oligomeric type cytokine receptor.

12. The chimeric receptor of claim 11, wherein the cytoplasmic domains of the first and second peptides are derived from an interleukin-2 receptor (IL-2R).

13. The chimeric receptor of claim 3, wherein the lymphocyte is a CD8<sup>+</sup> cytotoxic T lymphocyte (CTL).

14. The chimeric receptor of claim 13, wherein the CTL is normally dependent on cytokine B that is provided by helper T cells (T<sub>H</sub>), and wherein the chimeric receptor expressed in said CTL lessens the growth dependency of the CTL on T<sub>H</sub> cells in the presence of cytokine A.

15. The chimeric receptor of claim 14, wherein cytokine receptor B-R is an interleukin-2 receptor (IL-2R) and cytokine B is interleukin-2 (IL-2).





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16. The chimeric receptor of claim 15, wherein cytokine receptor A-R is selected from the group consisting of a granulocyte/macrophage colony stimulating factor receptor (GM-CSF-R), a  $\gamma$ -interferon receptor (IFN $\gamma$ -R) and an interleukin-3 receptor (IL-3R).

5        17. The chimeric receptor of claim 16, wherein cytokine receptor A-R is a granulocyte/macrophage colony stimulating factor receptor (GM-CSF-R) and cytokine A is granulocyte/macrophage colony stimulating factor (GM-CSF).

10       18. The chimeric receptor of claim 16, wherein cytokine receptor A-R is a  $\gamma$ -interferon receptor (IFN $\gamma$ -R) and cytokine A is  $\gamma$ -interferon (IFN $\gamma$ ).

19. The chimeric receptor of claim 16, wherein cytokine receptor A-R is an interleukin-3 receptor (IL-3R) and cytokine B is interleukin-3 (IL-3).

15       20. The chimeric receptor of claim 1, wherein both the cytoplasmic domain and the transmembrane domain are derived from cytokine receptor B-R.

21. The chimeric receptor of claim 3, wherein both the cytoplasmic domain and the transmembrane domain are derived from cytokine receptor B-R.

20

22. A recombinant polynucleotide comprising a region encoding a first peptide chain of claim 1.

25       23. A recombinant polynucleotide comprising a region encoding a second peptide chain of claim 3.

24. The recombinant polynucleotide of claim 22, further comprising a region encoding a second peptide chain of claim 3.

30       25. The recombinant polynucleotide of claim 22 in the form of an expression vector.

26. The recombinant polynucleotide of claim 24 in the form of an expression vector.

27. A cell containing an expression vector of claim 25.

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28. A cell containing an expression vector of claim 26.

29. The cell of claim 27, wherein the cell is a CD8<sup>+</sup> cytotoxic T lymphocyte (CTL).

5 30. The cell of claim 28, wherein the cell is a CD8<sup>+</sup> cytotoxic T lymphocyte (CTL).

31. A method of using a recombinant polynucleotide comprising a region encoding a first peptide chain, wherein the first peptide chain comprises an extracellular domain derived from cytokine receptor A-R that binds cytokine A, joined, via a transmembrane domain, to a cytoplasmic domain derived from cytokine receptor B-R that binds cytokine B, wherein  
10 cytokine B is a cytokine that is normally required by a lymphocyte for growth and proliferation and wherein the chimeric receptor expressed in said lymphocyte lessens the growth dependency of the lymphocyte on cytokine B in the presence of cytokine A, the method comprising transforming a cell with the recombinant polynucleotide.

15 32. The method of claim 31, wherein the recombinant polynucleotide is in the form of a recombinant expression vector.

20 33. The method of claim 32, wherein the cell is a lymphocyte.

34. The method of claim 33, wherein the lymphocyte is a CD8<sup>+</sup> cytotoxic T lymphocyte (CTL).

25 35. The method of claim 34, wherein cytokine receptor B-R is an interleukin-2 receptor (IL-2R) and cytokine B is interleukin-2 (IL-2).

30 36. The method of claim 35, wherein cytokine receptor A-R is selected from the group consisting of a granulocyte/macrophage colony stimulating factor receptor (GM-CSF-R), a  $\gamma$ -interferon receptor (IFN $\gamma$ -R), and an interleukin-3 receptor (IL-3R).

37. A cell produced by the method of claim 30 and progeny thereof.

38. A cell produced by the method of claim 31 and progeny thereof.

35 39. A cell produced by the method of claim 32 and progeny thereof.



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40. A cell produced by the method of claim 33 and progeny thereof.

41. A cell produced by the method of claim 34 and progeny thereof.

5

42. A cell produced by the method of claim 35 and progeny thereof.

43. A cell produced by the method of claim 36 and progeny thereof.



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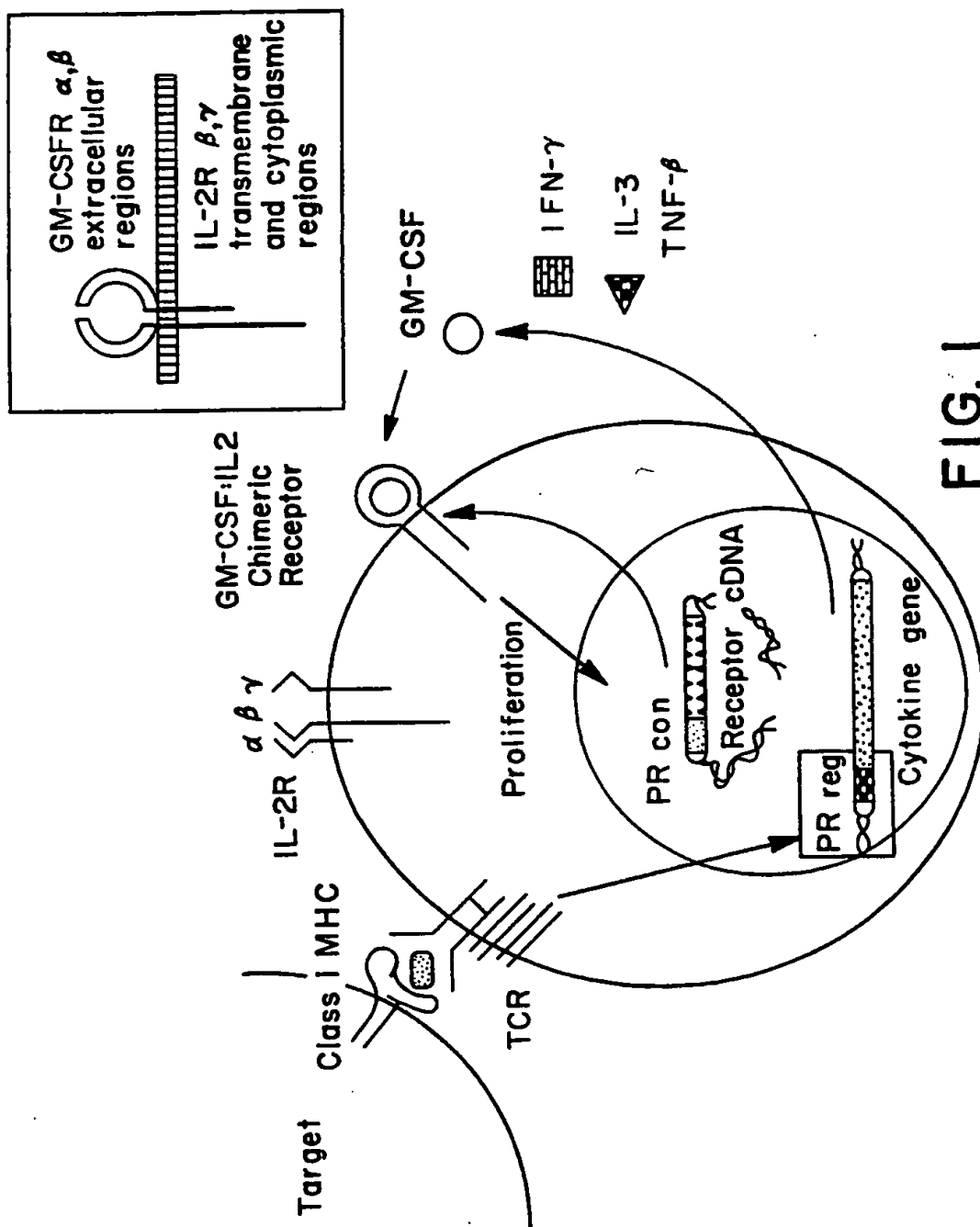


FIG. 1





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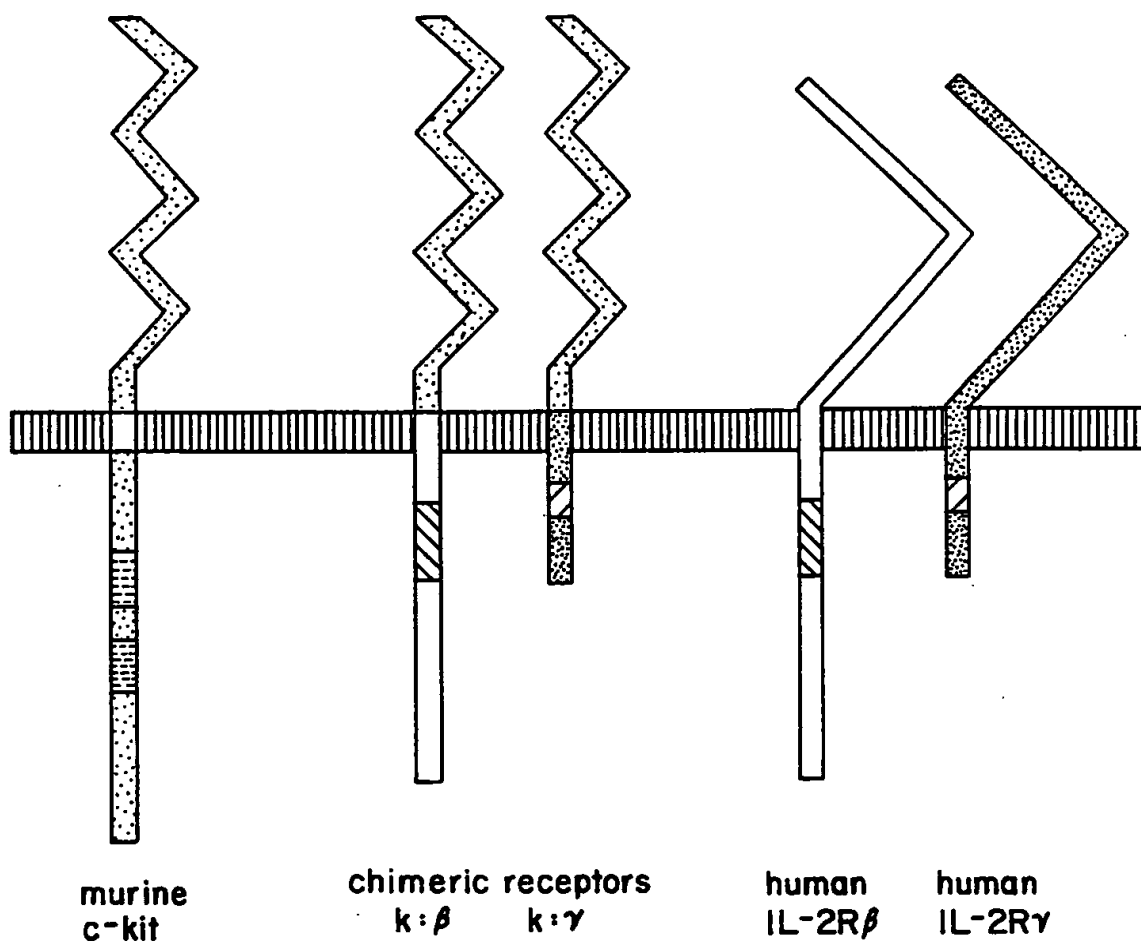
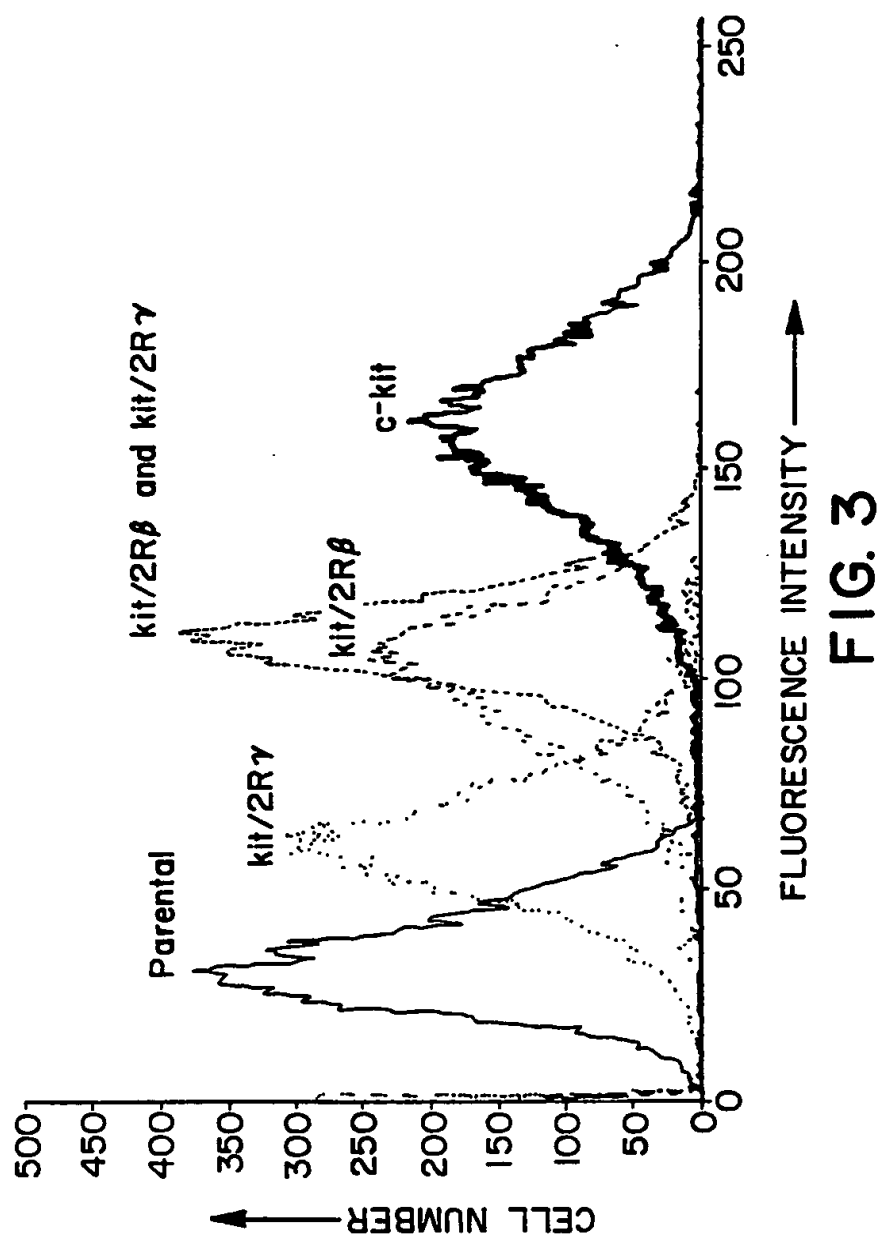


FIG. 2



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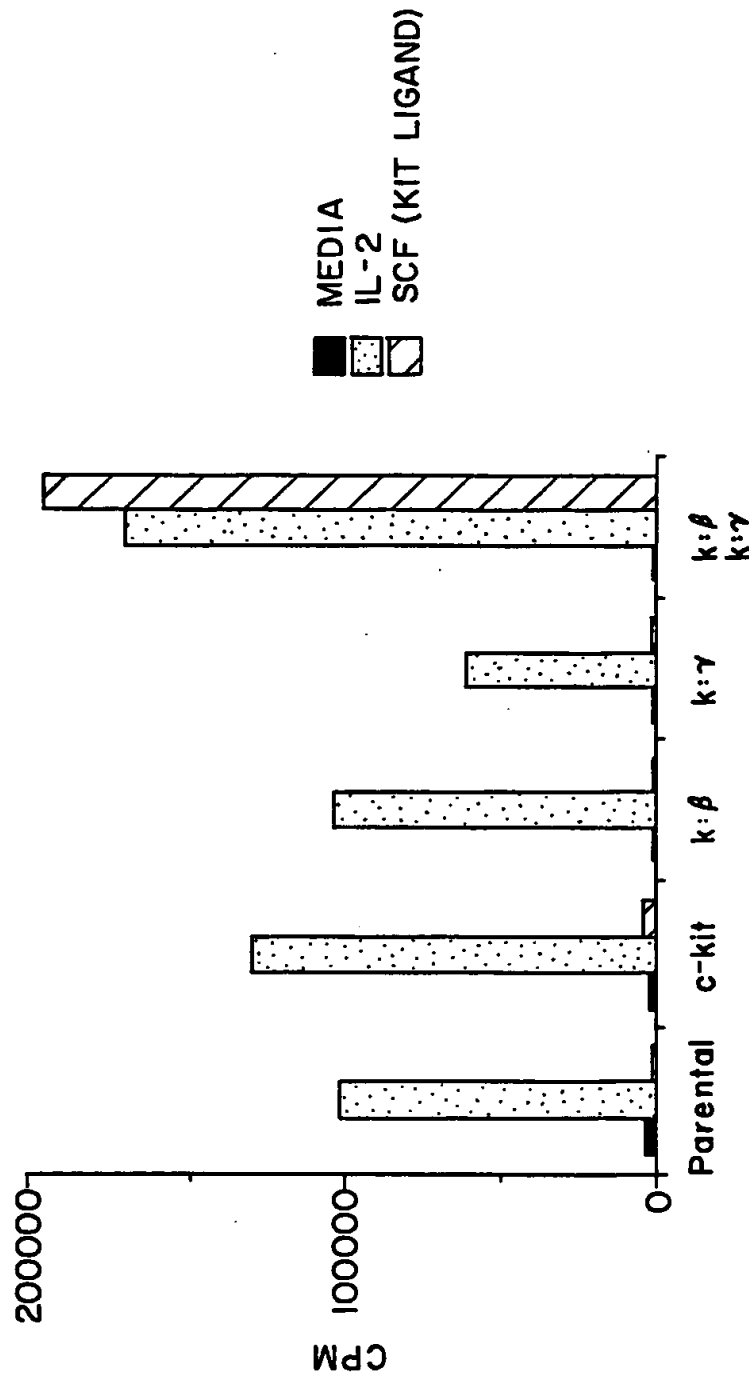


FIG. 4



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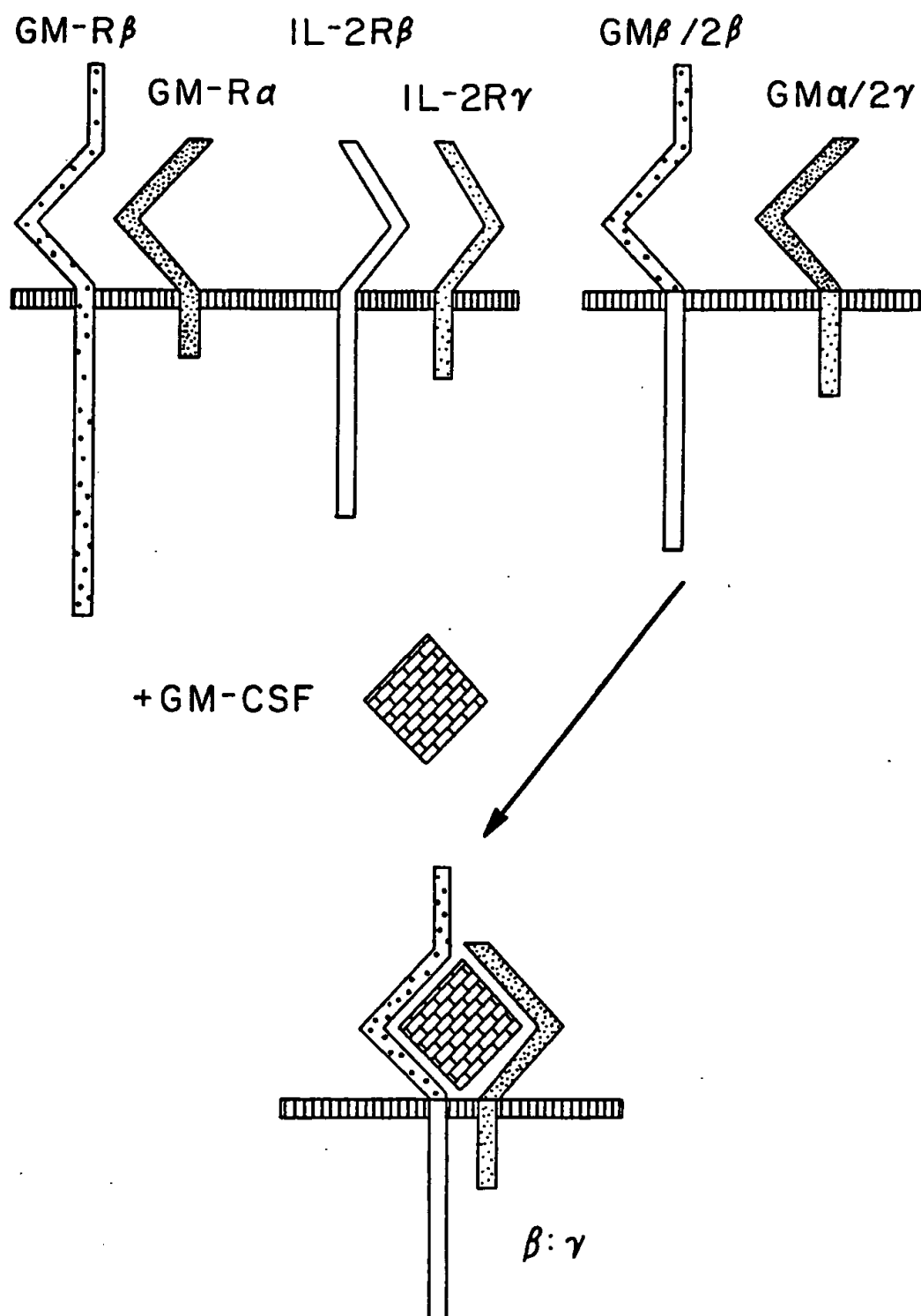


FIG. 5





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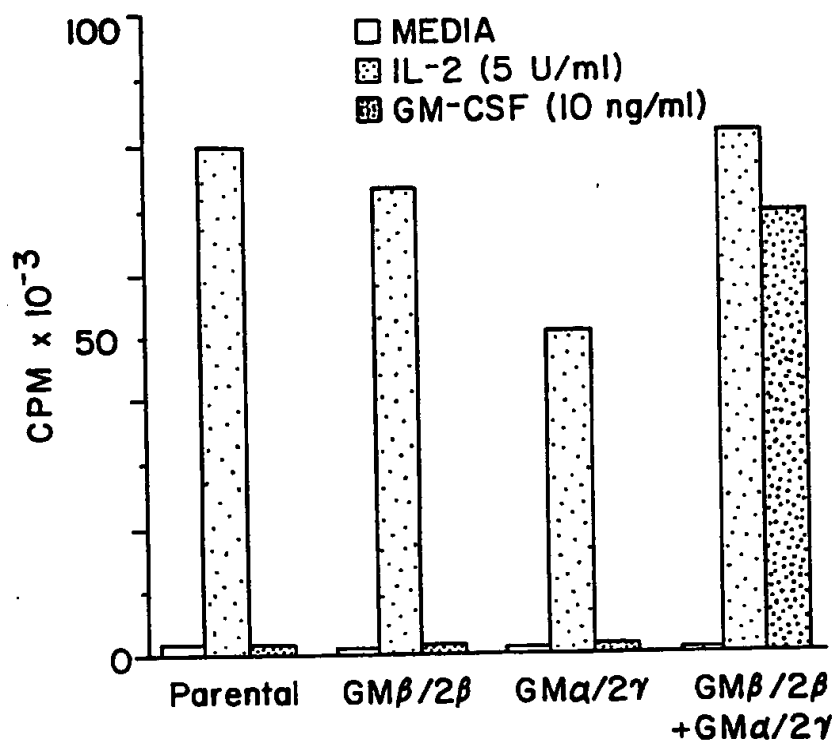


FIG. 6



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/03769

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C07K 13/00; C12N 15/12, 15/62

US CL : 435/69.7, 252.3, 320.1; 530/350; 536/23.4

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.7, 172.3, 252.3, 320.1; 530/350; 536/23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN/MEDLINE

search terms: {(fusion# or hybrid# or chimera#) (2a) (protein# or peptide# or polypeptide# or gene#)} (p) (receptor#)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,030,576 (DULL ET AL.) 09 July 1991, entire document.	1-43
Y	Journal of Experimental Medicine, Vol. 166, issued August 1987, M. Hatakeyama et.al., "TRANSMEMBRANE SIGNALING OF INTERLEUKIN 2 RECEPTOR", pages 362-375, see entire document.	1-43
Y	MOLECULAR AND CELLULAR BIOLOGY, Vol. 10, No. 5, issued May 1990, M.F. Roussel et.al., "Antibody-Induced Mitogenicity Mediated by a Chimeric CD2-c-fms Receptor" pages 2407-2412, see entire document.	1-43

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search

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Authorized officer

JOHN D. ULM

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## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	International Immunology, Vol. 3, No. 2, issued 21 September 1990, H. Mori et.al., "Signal transduction by interleukin 2 receptor beta chain: importance of the structural integrity as revealed by site-directed mutagenesis and generation of chimeric receptors", pages 149-156, see entire document.	1-43
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Vol. 190, No. 3, issued March 1993, K. Izuhara et.al., "THE CHIMERIC RECEPTOR BETWEEN INTERLEUKIN-2 RECEPTOR beta CHAIN AND INTERLEUKIN-4 RECEPTOR TRANSDUCES INTERLEUKIN-2 SIGNAL", pages 992-1000, see entire document.	1-43



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup>:</b> <b>C12N 15/62, C07K 15/00, C12N 15/85, 5/10, G01N 33/566</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 94/29458</b> <b>(43) International Publication Date:</b> 22 December 1994 (22.12.94)
<b>(21) International Application Number:</b> PCT/US94/06280 <b>(22) International Filing Date:</b> 3 June 1994 (03.06.94)  <b>(30) Priority Data:</b> 08/073,196      7 June 1993 (07.06.93)      US  <b>(71) Applicant:</b> AMGEN INC. [US/US]; Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US).  <b>(72) Inventors:</b> PACIFICI, Robert, Enrico; 914 Rosario Drive, Thousand Oaks, CA 91362 (US). THOMASON, Arlen, Read; 2298 Watertown Court, Thousand Oaks, CA 91360 (US). CHANG, Ming-Shi; 736 Calle Las Colinas, Newbury Park, CA 91320 (US).  <b>(74) Agents:</b> ODRE, Steven, M. et al.; Amgen Inc., Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US).	<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> HYBRID RECEPTOR MOLECULES  <b>(57) Abstract</b>  Provided are hybrid receptor molecules wherein one domain of the receptor is derived from the cytokine superfamily of receptors and other domain is derived from a heterologous family of receptors.		





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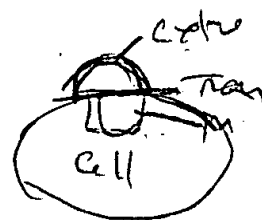


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## HYBRID RECEPTOR MOLECULES

Field of the Invention

5 This invention relates to biologically active hybrid receptor molecules. More specifically, the invention is directed to receptor molecules that are hybrids of a receptor extracellular domain and a receptor intracellular domain, where one of the domains  
10 is derived from certain members of the hematopoietic cytokine family of receptors, and the other domain is derived from an unrelated family of receptors.

Description of Related Art

15 Growth and differentiation of cells occurs by a variety of mechanisms. One common mechanism is via a cellular response to certain extracellular chemical or physical stimuli. Some of the chemical stimuli are  
20 known as ligands. Ligands bind to specific receptors on the cellular membrane, thereby ultimately resulting in the transition of a signal to the cell or other response.

The receptors are typically proteinaceous  
25 macromolecules that span a particular cell membrane. Most receptors possess three domains, the extracellular domain, the transmembrane spanning domain, and the intracellular domain. The extracellular domain of the receptor usually serves as the binding site for the  
30 ligand, the transmembrane or membrane spanning domain typically serves to anchor the receptor into the membrane, and the intracellular domain often serves to transmit a signal to the intracellular environment.

Signal transduction appears to occur in a variety  
35 of ways upon ligand binding, such as for example, by a conformational change in the structure of the receptor,



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by dimerization of two identical or related receptor-type molecules, or by internalization of the ligand (see, Schlessinger et al., *Neuron*, 9:383-391 [1992]; Vairo et al., *Immunol. Today*, 12:362-369 [1991]; Ullrich et al., *Cell*, 61:203-212 [1990]; Hatakeyama et al., *J. Exp. Med.*, 166:362-375 [1987]).

Many receptors have been identified, and the scientific literature has variously divided them into groups, superfamilies, families and/or classes of receptors based on common features such as tissue distribution of the receptors, nucleic acid or amino acid homology of the receptors, mechanisms of signaling by the receptors, or the type of ligand that binds to the receptors. A uniform system of classifying or grouping receptors however, has not been used in the literature.

One group of receptors has been termed the cytokine receptor superfamily. Most cytokines are soluble proteins that affect the growth and differentiation of many cell types such as cells involved in hematopoiesis and cells involved in the immune response. Cytokines exert their effect on the growth and differentiation of cells by binding to one or more members of this superfamily of receptors. The receptors are generally located on the cell surface membrane (plasma membrane). The cytokine receptor superfamily contains many families, such as, for example, the hematopoietic receptor family, the interferon receptor (IFN-R) family, the tumor necrosis factor receptor (TNF-R) family, the nerve growth factor receptor (NGF-R) family, the transforming growth factor (TGF) beta receptor family, and the interleukin-8 (IL-8) receptor family (see Taga et al. *FASEB J.*, 6:3387-3396 [1992]).

Another separate and distinct group of receptors is the protein-tyrosine kinase receptor family. This receptor family shares the common trait of



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phosphorylation (via ATP hydrolysis) of the hydroxyl group of a tyrosine residue of a selected intracellular enzyme, or autophosphorylation of the receptor itself upon ligand binding to the receptor. A large number of  
5 receptors have been identified as members of this family, including, among others, epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), and insulin receptor (IR).

Another distinct group of receptors is the atrial  
10 natriuretic peptide receptor family. This family of receptors has one or more of the atrial natriuretic peptides (ANPs) as ligands. ANP is involved in the regulation of fluids across the cellular plasma membrane. This family of receptors consists of atrial  
15 natriuretic peptide receptor A (ANPRA), atrial natriuretic peptide receptor B (ANPRB) and atrial natriuretic peptide receptor C (ANPRC). These receptors are each expressed at different levels in different types of cells (Wilcox et al., *Mol. Cell. Biol.*,  
20 11:3454-3462 [1991]).

Still another group of receptors are those with two common characteristics: they all have seven transmembrane spanning domains, and they are all believed to transduce signals to the intracellular  
25 environment via G-proteins (GTP binding proteins). This group includes such receptors as the rhodopsin and related opsin receptors, the alpha and beta adrenergic receptors, the muscarinic cholinergic receptors, and the yeast mating factor receptors. The thrombin receptor is  
30 also known to have seven transmembrane spanning domains (see WO 92/14750).

Many other receptor groups have also been identified, such as the steroid receptor family, of which the retinoic acid receptor is a member, the family  
35 of the endothelin receptors (Adachi et al., *FEBS Lett*, 311:179-183 [1992]) and related receptors, the glutamate





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family of receptors, and the family containing the transferrin receptor.

Several receptors have been cloned and the DNA sequences have been obtained. In addition, mutant  
5 receptors have been generated and tested for biological  
activity as compared to the naturally occurring, or  
wild-type, receptor molecules. For example, Quelle et al., (*Mol. Cell. Biol.*, 12:4553-4561 [1992]) have prepared mutants of the erythropoietin receptor. Mutant  
10 receptors are those in which one or more naturally occurring amino acids have been substituted or deleted, or those in which additional amino acids have been added. Some mutants are combinations of substitution, deletion, and/or insertion of amino acids.

15 One method of studying the mechanism(s) of receptor activation and signaling has been to construct artificial or synthetic receptor molecules. These molecules are generally known as hybrid or chimeric receptors. Such receptors typically possess the  
20 extracellular domain of one naturally occurring receptor and the intracellular domain of another naturally occurring receptor. The majority of hybrid receptors that have been generated ~~are intra-familial hybrids,~~  
i.e., the intra- and extracellular domains of the hybrid  
25 receptor are derived from members of the same family or superfamily of receptors.

Venkitaraman et al. (*Proc. Natl. Acad. Sci. USA*, 89:12083-12087 [1992]) describe hybrid receptor molecules between the CD8 receptor and the interleukin 7  
30 (IL-7) receptor, both of which are members of the cytokine superfamily of receptors.

Adachi et al. (*FEBS Lett.*, 311:179-183 [1992]) describe hybrid receptor molecules of the endothelin A and endothelin B receptors.

35 Koller et al. (*Mol. Cell. Bio.*, 12:2581-2590 [1992]) describe receptors that are hybrids of naturetic



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peptide receptor A (NPR-A) and natriuretic peptide receptor B (NPR-B), both of which are members of the guanylyl cyclase receptor family. In addition, hybrid receptors of NPR-A or NPR-B in combination with a portion of the epidermal growth factor receptor (EGFR) or the endotoxin receptor were generated by these researchers, but these hybrids were not stimulated by ligand.

Zon et al., (*Mol. Cell. Biol.*, 12:2949-2957 [1992]) discuss production of hybrid receptors between the erythropoietin receptor (EPOR) and the interleukin 3 (IL-3) receptor. Both of these receptors are members of the cytokine superfamily of receptors.

Fuh et al., (*Science*, 256:1677-1680 [1992]) describe a hybrid receptor between the extracellular domain of the human growth hormone receptor (hGHR) and the intracellular domain of murine granulocyte colony-stimulating factor receptor (G-CSFR). Both of these receptors are members of the cytokine receptor superfamily.

Seedorf et al., (*J. Biol. Chem.*, 266:12424-12431 [1991]) set forth the production of a hybrid receptor that consists of the extracellular domain of EGFR and the intracellular domain of the platelet derived growth factor receptor (PDGFR). Both of these receptors are members of the protein tyrosine kinase receptor family.

Lev et al. (*Mol. Cell. Biol.*, 10:6064-6068 [1990]) discuss a hybrid receptor between p145<sup>kit</sup>, a proto-oncogene, and EGFR. Both of these receptors are members of the protein tyrosine kinase receptor family.

Dull et al., U.S. Patent Nos. 4,859,609 (issued August 22, 1989) and 5,030,576 (issued July 9, 1991) describe hybrid receptors, and set forth specific hybrids between EGFR and the insulin receptor (IR), and between EGFR and HER2-erbB2, an oncogene. EGFR, IR, and



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HER2-erbB2 are all members of the protein-tyrosine kinase receptor superfamily.

Patent Cooperation Treaty WO 91/06570, published May 16, 1991, sets forth hybrid molecules between the  
5 antibody Fc receptor and an antibody. The hybrid molecule is preferably in a soluble form.

Some inter-familial, or heterologous, hybrid receptors have also been generated. Only a few of these have been found to be biologically active, i.e., are  
10 capable of transducing a signal from the extracellular environment to the intracellular environment. For example, Yan et al. (*Science*, 252:561-563 [1991]) describe a hybrid receptor molecule containing the  
15 extracellular domain of EGFR and the intracellular and transmembrane domains of the human low-affinity nerve growth factor receptor (NGFR). The hybrid was found to induce neurite outgrowth in cells stimulated with EGF; the hybrid was also able to specifically induce a NGF-responsive gene called transin.

20 Bernard et al. (*Proc. Natl. Acad. Sci. USA*, 84:2125-2129 [1987]) describe a hybrid receptor of the extracellular domain of interleukin 2 (IL-2) and the transmembrane and intracellular domains of EGF. Cells transfected with this hybrid did not respond to ligand  
25 added to culture medium, suggesting that the hybrid receptor was not biologically active.

Interfamilial hybrid receptors provide a means for obtaining information about newly identified receptors with unknown ligands. For example, the extracellular  
30 domain of a newly identified receptor may be linked to the intracellular domain of a receptor with a known signal transduction mechanism. Various potential ligands can then be tested to identify those that bind to the extracellular domain of the hybrid and are  
35 capable of transmitting a signal.



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There is a need in the art to provide a means of identifying the ligands of newly discovered receptors.

There is a further need in the art to provide hybrid receptors that can be used to increase or decrease

5 cellular responses to certain ligands through the use of agonists and/or antagonists to these receptors.

Accordingly, it is an object of the present invention to provide a hybrid receptor molecule wherein one domain of the hybrid receptor is derived from the  
10 cytokine superfamily of receptors, and the other domain of the receptor is derived from a separate and distinct or heterologous family of receptors. Various potential ligands for the molecule can be evaluated for their ability to stimulate or inhibit signaling to the  
15 intracellular environment.

#### SUMMARY OF THE INVENTION

This invention is based on the unexpected discovery  
20 that hybrid receptors comprising an extracellular domain from certain members of one receptor family and an intracellular domain from certain members of a heterologous receptor family possess biological activity when DNA encoding the hybrid receptor is transfected  
25 into and expressed in cell lines.

In one aspect, this invention provides a biologically active hybrid receptor molecule, wherein one domain of the hybrid is a member of the hematopoietic cytokine receptor family, and the other  
30 domain is a member of a separate and distinct family of receptors.

In another aspect, the invention provides a hybrid receptor molecule wherein the extracellular domain is a member of the protein-tyrosine kinase receptor family  
35 such as epidermal growth factor receptor (EGFR) and the transmembrane and intracellular domains are members of





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the hematopoietic cytokine receptor family such as erythropoietin receptor (EPOR).

In yet another aspect, the invention provides a hybrid receptor molecule wherein the extracellular domain is a member of the hematopoietic cytokine receptor family such as EPOR and the transmembrane and intracellular domains are members of the atrial natriuretic peptide receptor (ANPR) family such as ANPRA, ANPRB and ANPRC.

In one other aspect, the invention provides host cells transfected with a DNA sequence encoding the hybrid receptor and expressing a biologically active form of the hybrid receptor on a particular cell membrane.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the erythropoietin receptor (EPOR), the epidermal growth factor receptor (EGFR), and two EGFR-EPOR hybrid receptors called EECA and EECB (described in detail in Example I), that were constructed using the extracellular domain of EGFR and various fragments of the transmembrane and intracellular domains of EPOR. The darkened regions represent EPOR sequences, the darkened vertical bar represents the transmembrane domain, the open regions represent EGFR sequences, the striped region represents the tyrosine kinase domain of EGFR, and the dotted region represents the WSXWS motif of EPOR. The numbers above each receptor construct represent the number of amino acids from that domain that were used in the construct. Negative numbers refer to the signal sequence; number 1 is the first amino acid at the amino terminus of the mature receptor sequence.



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Figure 2 depicts the sustained growth of murine 32D cells transfected with and expressing various receptor-DNA constructs, and grown in the presence of the growth factors IL-3, EGF, TGF-alpha, or EPO. Darkened squares represent IL-3 (interleukin-3), darkened triangles represent EGF (epidermal growth factor), inverted triangles represent TGF-alpha (transforming growth factor alpha), open circles represent EPO (erythropoietin), and the solid line represents no added growth factor.

#### DETAILED DESCRIPTION OF THE INVENTION

The following terms are used to describe the invention.

The term "receptor" refers to a molecule, typically composed primarily of protein, that is associated, at least transiently, with one or more types of cellular membranes, and has as its main biological function the ability to bind a specific ligand or group of ligands, and, upon ligand binding, to mediate signal transduction, either directly or indirectly, in the cell. The cell from which the receptor is obtained may be any vertebrate cell, invertebrate cell, plant cell, bacterial cell or any other microorganism cell. The receptor may also reside naturally on the coat of any virus. The cellular membrane from which the receptor is obtained may be the plasmalemma (the membrane surrounding the cell) or any intracellular membrane surrounding any cellular organelle, such as the mitochondrial membrane, the chloroplast membrane (inner or outer), the nuclear membrane, the lysosomal membrane, the vacuolar membrane (the tonoplast), the endoplasmic reticulum, and the like. The typical receptor has three portions or domains, namely, an intracellular domain, an extracellular domain, and a transmembrane domain. In



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addition, the receptor typically has a sequence of about 5-25 amino acids at its amino terminus that serve to target the receptor to the proper membrane. These domains will vary in size and in function from receptor to receptor. Typically, the extracellular domain binds to one or more ligands, the transmembrane domain anchors the receptor into the membrane, and the intracellular domain perceives the binding of ligand and transmits a signal to the interior of the cell (the intracellular environment). Usually, the transmembrane domain of the receptor is comprised primarily of hydrophobic amino acids, while the extracellular domain and the intracellular domain may contain all types of amino acids.

The term "hybrid" refers to the amino acid composition and/or DNA sequence of the receptor. The receptors of this invention are typically combinations of pieces or fragments of naturally occurring receptors, and/or mutants thereof, however the hybrid may be comprised of full-length sequences of any or all of the domains. Typically, the hybrid receptor will be comprised of an extracellular domain, a transmembrane domain, and an intracellular domain. The extracellular domain will be derived from certain members of one family of receptor molecules, while the intracellular domain will be derived from certain members of a second family of receptor molecules. The transmembrane domain may be derived from the same receptor as either the intracellular or extracellular domain, or it may be derived from a third receptor source. In addition, any or all of the domains may be synthetic in origin, i.e., based on sequences that are not naturally occurring.

The terms "heterologous", "heterologous receptor", and "heterologous receptor domain" refer to receptors or receptor domains derived from separate and distinct groups, classes, families or superfamilies of receptors.



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The hybrid receptor molecules of this invention are heterologous in that one domain of the hybrid receptor is typically derived from one family of receptors, such as for example, the hematopoietic cytokine receptor family, while another domain of the receptor is derived from an unrelated receptor family, such as, for example, the protein-tyrosine kinase receptor family.

The terms "family", "class", and "superfamily" refer to a collection of cell membrane receptors that are considered to have a certain level of homology, either in terms of structure (e.g., a certain level of amino acid or nucleic acid sequence homology), function (e.g., they are all involved in antigen recognition or bind a certain type of ligand), or activity (e.g., they all hydrolyze ATP). Typically, superfamilies comprise more than one class or family of receptors.

The terms "hematopoietic cytokine receptor" and "hematopoietic cytokine receptor family" refer to receptors that have one or more cytokines as their primary ligand(s), although they may have different mechanisms of signaling. As used herein, cytokines are defined as molecules usually comprised primarily of protein, that affect growth and/or differentiation of various cells. Many of the hematopoietic cytokine receptors contain the WSXWS amino acid sequence motif (W represents tryptophan; S represents serine, and X represents a nonconserved amino acid).

The term "ligand" refers to a molecule that binds to a receptor with a certain specificity and affinity. The ligand(s) may be natural or synthetic, and the ligand(s) may have the ability to bind to more than one type of receptor. The ligand may be an inorganic or an organic molecule. The organic molecules may be composed of nucleic acid, protein, lipid, carbohydrate, or any other type of organic molecule combination thereof.





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The term "biologically active" refers to hybrid receptors that are (1) capable of binding one or more ligands, and (2) able to respond to the binding by signaling the cell, either directly or indirectly in a manner that is detectable and is distinct from the response of cells not transfected with DNA encoding the hybrid receptor. The response of the receptor to the ligand binding will be detectable by assaying for signaling, such as by a conformational, chemical, or structural change in the receptor (for example, phosphorylation of the receptor), dimerization of the receptor with another molecule, production of a chemical messenger on the surface of or inside of the cell (such as cGMP), immunological detection, growth and/or differentiation, or other assay that is appropriate for the particular hybrid receptor being evaluated.

#### Methods of Making the Invention

##### 20        1. Selection of Receptor Extracellular Domain

The extracellular domain of the hybrid receptor may be any naturally occurring amino acid sequence, or a synthetic amino acid sequence that is known or is believed to be the extracellular domain of a receptor. Such amino acid sequences are encoded by naturally occurring or synthetic DNA sequences. In addition, the extracellular domain may be one or more fragments or pieces of receptor extracellular domain sequences derived from more than one family of receptors. Included within the scope of this invention are newly identified sequences believed to be receptor or membrane bound sequences with no known function.

The extracellular domain may be derived from any bacterial receptor, vertebrate or invertebrate receptor, plant receptor, or a receptor from any other source; the receptor may naturally be found on the plasmalemma of a



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certain cell type(s) or on a membrane that surrounds a cellular organelle such as, for example, the endoplasmic reticulum, the nucleus, a lysosome, a vacuole, a mitochondrion, or a chloroplast. The extracellular receptor amino acid sequence may be a fragment and/or a mutant form of the known sequence from which it is derived. For purposes herein, a mutant is defined as a polypeptide encoded by a DNA sequence containing any alteration in the native DNA sequence, whether it be nucleotide insertions, deletions, or substitutions. In addition, changes in the carbohydrate composition of the polypeptide (including alterations of the sugar residues of the carbohydrate linkage, and/or addition or subtraction of carbohydrate moieties on the polypeptide as compared with the native sequence) are considered herein to be mutations.

The extracellular domain may be a hybrid molecule in and of itself, i.e., it may be composed of fragments or sequences derived from more than one receptor extracellular domain.

Where the extracellular domain is a fragment of an extracellular domain full length sequence, the fragment will typically include the known or putative ligand binding region of the extracellular domain, as well as any other region of the extracellular domain that is believed to be at least partially responsible for the biological activity of the receptor from which it is derived.

Typically, the extracellular domain will contain, usually at its amino terminus, a sequence of about 5-25 amino acids that direct the receptor molecule to the proper membrane after the receptor is synthesized in the cell. Such sequences usually are known as signal sequences or leader peptides.

This invention contemplates the use of primarily two types of extracellular domains: those with known



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ligands, and those with no known ligands. Production of hybrid receptors that have extracellular domains with known ligands will be useful for a variety of functions, but especially for screening new ligands that are  
5 believed to either enhance the level of intracellular signaling, or to decrease or inhibit intracellular signaling.

Production of hybrid receptors with extracellular domains for which no known function and/or ligand exists  
10 will be useful for identifying the ligands and/or functions of the novel extracellular domain(s). Once the ligands have been identified, one may obtain information on the receptor's inherent activity. In addition, novel ligands that bind to the receptor then  
15 can be screened to evaluate their potential for increasing or decreasing receptor activity.

Typically, the extracellular domain will be selected from a receptor whose inherent activity either confers a beneficial or a detrimental function upon  
20 cellular homeostasis; the objective will be to identify ligands that increase the activity of this receptor thereby enhancing the beneficial effects of the receptor, or decrease its activity where the receptor's activity is known to be or is believed to be detrimental  
25 to the cell.

Preferred extracellular domains of this invention are those with no known function and/or ligand, but with some homology, either at the nucleic acid or amino acid level, to any domain of a known receptor. By way of  
30 example, a preferred extracellular domain is encoded by a cloned DNA sequence of unknown function that has sequence homology with the DNA encoding the intracellular kinase domain of a protein tyrosine kinase receptor, or a cloned DNA sequence that has sequence  
35 homology with the WSXWS motif that is present in many members of the cytokine receptor family. Other



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preferred extracellular domains of this invention include those of the protein tyrosine kinase family such as epidermal growth factor receptor and its homologs, the erythropoietin receptor and its homologs, other  
5 members of the hematopoietic cytokine receptor family and homologs, the atrial natriuretic peptide receptors, such as ANPRA (Lowe et al., *EMBO J.*, 8:1377-1384 [1989]), ANPRB (Chang et al., *Nature*, 341:68-72 [1989]), or ANPRC, and their homologs, granulocyte-colony  
10 stimulating factor receptor and its homologs, and human fetal liver kinase-2 receptor and its homologs.

If the intracellular domain of the hybrid receptor is not a member of the hematopoietic cytokine receptor family, then the extracellular domain will be selected  
15 from this family.

## 2. Selection of Receptor Intracellular Domain

The intracellular domain of the hybrid receptor will be obtained from a receptor family that is separate  
20 and distinct from the extracellular domain receptor family. Typically, this domain will be selected based on its ability to produce or to transmit a detectable response in cells expressing this domain when ligand is added to the cell culture medium. However, the  
25 intracellular domain from any receptor may be used. The intracellular domain may be derived from a bacterial receptor, a vertebrate or invertebrate receptor, a plant receptor, or a receptor from any other source; the receptor may naturally be found on the plasmalemma of a  
30 certain cell type(s) or on a membrane that surrounds a cellular organelle such as, for example, the endoplasmic reticulum, the nucleus, a lysosome, a vacuole, a mitochondrion, or a chloroplast. The intracellular receptor DNA and/or amino acid sequence may be a  
35 fragment and/or a mutant form of the known sequence from which it is derived. For purposes herein, a mutant is





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defined as a polypeptide encoded by a DNA sequence containing any alteration in the native DNA sequence, whether it be nucleotide insertions, deletions, or substitutions. In addition, changes in the carbohydrate composition of the polypeptide (including alterations of the sugar residues of the carbohydrate linkage, and/or addition or subtraction of carbohydrate moieties on the polypeptide as compared with the native sequence) are considered to be mutations.

10       The intracellular domain may be a hybrid molecule in and of itself, i.e., it may be composed of fragments or sequences derived from more than one receptor intracellular domain, provided that the domain is constructed in such a manner as to be functional in signal transduction.

15       Preferred intracellular domains are those with a known and assayable signal transduction mechanism or activity such as, for example, the erythropoietin receptor intracellular domain, the granulocyte-colony stimulating factor receptor, the granulocyte macrophage colony stimulating factor receptor, the epidermal growth factor receptor intracellular domain, or the atrial natriuretic peptide receptor type A (Lowe et al., *EMBO J.*, 8:1377-1384 [1989]), type B (Chang et al., *Nature*, 20   341:68-72 [1989]), or type C intracellular domain.

25       If the selected extracellular domain of the hybrid receptor is not a member of the hematopoietic cytokine receptor family, then the intracellular domain of the hybrid receptor will be selected from the hematopoietic cytokine receptor family. Where the extracellular domain has no known activity, and thus it is unclear whether it is a member of the hematopoietic cytokine receptor family, the intracellular domain may be a member of this family.

35



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### 3. Selection of Receptor Transmembrane Domain

The transmembrane domain sequence of the hybrid receptor may be obtained from any source. Typically however, it will be selected from the same receptor as either the intracellular domain or the extracellular domain. However, the transmembrane domain may also be selected from a receptor that is a member of a separate and distinct group from either the extracellular domain receptor or the intracellular domain receptor. While the main purpose of the transmembrane domain appears to be to anchor the receptor into the membrane, this domain may also be important in certain receptors for signal transduction. Thus, it may be necessary that the transmembrane domain be of the same origin as either the extracellular domain or intracellular domain, depending on the predicted or known mechanism of signaling.

### 4. Hybrid Receptor Preparation

The hybrid receptors of this invention are typically prepared using recombinant DNA technology. A DNA construct containing the DNA sequences of the selected intracellular, extracellular, and transmembrane domains is prepared, usually by isolating the desired cDNA sequences for each domain of the hybrid receptor, using methods well known in the art. These methods include, without limitation, polymerase chain reaction (PCR) which is particularly useful where at least a partial sequence of the gene of interest is known; and cDNA and/or genomic library screening with suitable probes (usually oligonucleotides and/or antibodies for cDNA libraries, and oligonucleotides or cDNA sequences for genomic libraries). Some of these methods as well as other methods useful for molecular cloning are set forth by Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY [1989]).



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After the DNA sequences for each of the domains have been obtained in suitable quantities, they are ligated in the proper orientation, thereby producing a single DNA construct encoding the intracellular, transmembrane, and extracellular domains of the desired hybrid receptor. The ligation may be done with several DNA fragments simultaneously, or it may be done in successive steps. In addition, one fragment of DNA may first be ligated into a vector, after which time the other fragment(s) of DNA are then ligated into the same vector at the correct position.

In some cases, it may be necessary to first make the ends of each DNA fragment compatible for ligation to each other. This is done by either blunting the ends of each of the DNA fragments, or ~~cutting the ends with~~ appropriate restriction endonucleases. Both of these methods are described in Sambrook et al., *supra*. This single construct is then ligated into a suitable vector (unless the DNA fragments of interest were previously ligated into the vector one at a time) for transfection into selected cells.

The DNA encoding the hybrid receptor will typically be placed into a vector for amplification and for expression in the host cells. Any eukaryotic expression vector may be used when the hybrid receptor is to be expressed in eukaryotic cells. Prokaryotic expression vectors will be used for expression in bacterial cells. Selection of the expression vector will depend on several factors such as the choice of restriction endonuclease sites in the polylinker region of the vector, the type of promoter, and the selectable marker. Preferred promoters are those that yield a high level of transcription in a variety of host cells such as a retrovirus promoter (e.g. the cytomegalovirus promoter). Preferred selectable markers are neomycin, hygromycin, ampicillin, tetracycline, and other antibiotic



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resistance markers. Preferred vectors are pRc/CMV and pRc/RSV (both available from InVitrogen, San Diego, CA), pXT 1 (Stratagene, San Diego, CA) and pLJ (Korman et al., *Proc. Natl. Acad. Sci. USA*, 84:2150-2154 [1987]).

5 While recombinant DNA methods are the usual means for preparing hybrid receptors, other methods useful for preparation of these receptors may be employed as well. Such methods include, for example, chemical and/or enzymatic synthesis of either the amino acid or DNA  
10 sequence of the hybrid receptor, using methods well known in the art.

#### 5. Expression of the Hybrid Receptor

The hybrid receptor DNA vector construct may be  
15 transfected into a selected cell line for expression and evaluation of receptor activity. Transfection can be accomplished using any known method, including without limitation, the calcium phosphate procedure, electroporation, viral infection (via the use of  
20 retroviruses), lipofection, DEAE-dextran, or microinjection. The transfection method used will depend in part on the cell type being transfected. For bacterial cells, electroporation is generally preferred. For mammalian cells, transfection can be accomplished  
25 using electroporation, or alternatively, the DEAE-dextran method as described in Section 9.2 of Ausubel et al., eds. (*Current Protocols in Molecular Biology*, Greene and John Wiley and Sons, N.Y. [1987]).

Selection of host cell lines for incorporation and  
30 expression of the hybrid receptor DNA will normally depend on the hybrid receptor to be evaluated. In some cases, the cell line selected will be one that does not express, at very high levels, the naturally occurring receptors from which the hybrid was constructed, and  
35 preferably one that does not express such receptors at all. In addition, the selected cell line preferably





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will be one that does not naturally produce a significant amount of the ligand or ligands to be screened. In some cases, i.e., where growth is the end-point of the signal transduction process, the preferred  
5 cell line will be one that is dependent for growth and/or survival on one or more growth factors that the cells do not produce endogenously and that are not present in serum, but that can be added exogenously to the cell culture medium. In this type of system, the  
10 factor can be removed from the cell culture medium, and then only cells stimulated by the hybrid receptor ligand will grow.

Preferred cell lines for use in this invention are murine 32D cells (ATCC No.CRL 11346, deposited May  
15 13, 1993 with the American Type Culture Collection ["ATCC"], 12301 Parklawn Drive, Rockville, MD 20852, USA), COS-1 and COS-7 cells (African Green monkey kidney cells, ATCC Nos. CRL 1650 and CRL 1651, respectively), CBT6 cells (Pan et al., *Virology*, 125:1-7 [1983]), TF-1  
20 cells (Kitamura et al., *J. Cell. Physiol.*, 140:323-334 [1989]), FDC-P1 cells (Spooncer et al., *Nature*, 310:228-230), HEL cells (human erythroleukemia cells, ATCC No. TIB 180), and Ba/F3 cells (Palacios et al., *Cell*, 41:727-734).

25 In addition to expressing the hybrid receptor molecules in cultured cells, the receptors may be expressed *in vivo* in a variety of animals such as mice or other rodents, using standard procedures known in the art such as those set forth in Hogan et al., eds.,  
30 *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY [1986]).

#### 6. Hybrid Receptor Activity Assays

35 After transfection of the host cell line, the cells can be screened for incorporation of the hybrid receptor



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DNA into the nucleus and/or for expression of the hybrid receptor on the cell membrane.

Incorporation of the hybrid receptor DNA into the nucleus can be analyzed by Southern blotting total  
5 nuclear DNA of the host cell, and probing this blot with a probe designed to specifically detect a portion of the hybrid receptor DNA sequence. Other methods of  
10 detecting incorporation of DNA into the nucleus include, for example, polymerase chain reaction (PCR), and probe protection using a radioactive DNA or RNA probe that is hybridized to the DNA of interest on a Southern blot.

Expression of the hybrid receptor polypeptide can be evaluated in a variety of ways. To measure the level  
15 of hybrid receptor protein in the cell, an antibody directed against particular regions of the hybrid receptor may be used in either a Western blot analysis or in an immunoprecipitation analysis. Expression may  
20 also be monitored using fluorescence activated cell sorting (FACS). Alternatively, or additionally, bioassays to detect the activity of the hybrid receptor  
25 may be used. Here, the ligand or suspected ligand is added to the cell culture along with other reagents as necessary for analyzing receptor activity (for example,  $^{32}\text{P}$ -ATP,  $^3\text{H}$ -thymidine,  $^{32}\text{P}$ -GTP, and the like); after an  
30 appropriate period of time, the cells are assayed for certain changes that may have occurred in response to ligand binding. Some of these changes may include for example, phosphorylation of the receptor itself or of another protein, production of cGMP or cAMP, or  
35 expression of particular genes in the cell. In addition, the rate of host cell proliferation or the rate of host cell death may be a means of measuring hybrid receptor activity.

To measure the level of hybrid receptor mRNA in the  
35 host cell, Northern blot analysis, RNase protection



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assays, and/or reverse transcriptase/PCR assays can be conducted.

#### 7. Screening for Receptor Ligands

5 One key feature of the hybrid receptors provided in this invention is their use in screening for novel ligands that may either increase or decrease the level of signaling in the cell. In addition, these receptors can provide a means of producing a signal in a cell that  
10 might not normally receive a signal in response to a certain ligand.

The ligand(s) to be tested can be added to the transfected host cell culture media at several concentrations over various periods of time, and binding  
15 can be assessed by the use of one or more assays designed to detect ligand binding as discussed above.

Where the hybrid receptor is expressed in transgenic mammals, the ligand to be evaluated would be administered to the mammal over a wide range of doses.  
20 The effects of the ligand on the mammal could then be tested using suitable *in vitro* assays (by extracting and analyzing tissues expressing the hybrid receptor) or by *in vivo* evaluations.

The invention will be more fully understood by  
25 reference to the following examples. These examples should not be construed in any way as limiting the scope of the invention.

#### EXAMPLE I: PRODUCTION OF AN EGFR-EPOR HYBRID RECEPTOR

30

##### 1. Cell preparation

Cell cultures of the murine cell line 32D clone 3 (deposited with the ATCC as accession no ATCC# CRL 11346, deposited May 13, 1993) were used in this study.  
35 This cell line was selected for its dependence for growth and survival on interleukin-3 (IL-3). In



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addition, this cell line does not endogenously produce detectable levels of either the erythropoietin receptor (EPOR) or the epidermal growth factor receptor (EGFR).

The cells were cultured in Standard Medium  
5 consisting of RPMI 1640 medium (Gibco/BRL, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT) and 100 pg/ml recombinant murine interleukin 3 (IL-3; Peprotech, Rockyhill, NJ).  
10 The cultures were kept in an incubator at 37° C and 5% CO<sub>2</sub>, and were routinely passaged by dilution into fresh medium about once per week to maintain the density between 10<sup>4</sup> and 10<sup>6</sup> cells/ml.

## 2. DNA Constructs

15 Two EGFR/EPOR hybrid receptor DNA constructs were prepared. The receptors encoded in these constructs are depicted in Figure 1. Both of the hybrids contained the extracellular domain of EGFR from amino acids -24 to 620 (where -24 through -1 are the signal sequence amino  
20 acids for EGFR; see Lin et al., *Science*, 224:843-848 [1984]). One of the hybrids, called EECA, contained the transmembrane and cytoplasmic domain of murine EPOR, spanning from amino acids 225-483 of EPOR. The other hybrid, EECB, contained a larger portion of the murine  
25 EPOR sequence and included the highly conserved amino acid motif WSXWS (W = tryptophan; S = serine; X is any amino acid), which is the first portion of the extracellular domain of EPOR. This hybrid receptor contained amino acids 201-483 of EPOR.

30 Human EGFR cDNA was obtained from a human placenta library prepared in the pSPORT vector (Gibco/BRL, Grand Island, NY) by probing the library with a 400 base pair cDNA probe. The cDNA probe was obtained by PCR amplification of a 400 base pair fragment of an EGFR  
35 cDNA sequence. The PCR probes were selected based on





- 24 -

the published EGFR sequence (Lin et al., *Science*, 224:843-848 [1984]). These probes are set forth below:

Probe 1 (SEQ ID NO:1):

5

5'-AAGATCAAAGTGCTGGGCTCCGGT-3'

Probe 2 (SEQ ID NO:2):

10

5'-ATGGTATTCTTTCTCTTCCGC-3'

The murine EPOR full length cDNA sequence was obtained as follows: Human fetal liver mRNA (Clonetech, Palo Alto, CA, catalog number 6527-2) was reverse transcribed into cDNA using reverse transcriptase and a primer based on the published sequence of human EPOR (Jones et al., *Blood*, 76:31-35 [1991]). The cDNA was amplified using PCR and specific primers for the 3' and 5' regions of the cDNA based on the same EPOR published sequence. The EPOR sequence was inserted into the vector PRC/CMV (InVitrogen Corp, San Diego, CA; catalog number V750-20).

The EECA construct was prepared using a two step PCR (polymerase chain reaction) technique as described by Higuchi (*PCR Protocols: A Guide to Methods and Applications*, M. Innis et al., eds. Academic Press, NY, pp. 177-183 [1989]). For all PCR reactions, 75 ng of cDNA template was added to 100 µl of PCR reaction mix containing 1 unit of Deep vent polymerase (New England Biolabs, Beverly, MA), 1 X Deep vent buffer, 200 µM of each nucleotide triphosphate and 20 pmol of each primer. Each reaction was cycled 20 times.

The first step of this process was conducted to obtain the appropriate cDNA fragments of both EGFR and EPOR. The following primers were used with EGFR cDNA as



- 25 -

a template to obtain the EGFR extracellular domain linked to a portion of EPOR:

Primer A (SEQ ID NO:3):

5

5'-GCCAACGCCACAACCACCGCGCGCGGCCGCTGACTCCG-3'

Primer B (SEQ ID NO:4):

10

5'TGAGAGACAGCGTCAATATTAGCGGGATCTTAGGCCCATT-3'

Primer A corresponds to nucleotides -86 to -48 of the coding strand of EGFR with the exception of nucleotides -58 and -64 which were changed from C to G to generate a *NotI* restriction site. Primer B corresponds to nucleotides 768-747 of the non-coding strand of EPOR and nucleotides 1932-1915 of the non-coding strand of EGFR, with the exception of nucleotides 749 and 752 which were both changed to A to generate a *SspI* restriction site. The cycle sequence for PCR was 96°C for 15 sec., 68°C for 30 sec., and 72°C for 2 minutes.

To obtain the appropriate portion of EPOR intracellular domain cDNA for this hybrid receptor, the following two primers were used in the first step of the PCR process:

Primer C (SEQ ID NO:5):

30

5'-AATGGGCCTAAGATCCCGCTAATATTGACGCTGTCTCTCA-3'

Primer D (SEQ ID NO:6):

5'-AGCAGCCACAGCTGGAAGTTAC-3'

35



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Primer C is the complement to primer B, and primer D corresponds to EPOR sequence downstream of a unique *BglIII* restriction site. For generation of this DNA fragment, the PCR cycle sequence was 96°C for 15 sec., 64°C for 30 sec., and 72°C for 1 minute.

The DNA fragments from each of the above PCR reactions were purified using standard agarose gel electrophoresis methods for extraction and purification. The second step of the two-step PCR process was then conducted with these DNA fragments and primers A and D above. The PCR cycle sequence for this step was 96°C for 15 sec., 64°C for 30 sec., and 72°C for 2 minutes.

The EECB construct was prepared using the same two-step PCR procedure (Higuchi, *supra*) under the same reaction conditions as set forth above. Four primers were used to generate the appropriate cDNA fragments.

To obtain the EGFR extracellular domain sequence, primer A (set forth above) was used with primer E and the EGFR cDNA for PCR amplification.

Primer E (SEQ ID NO:7)

5'-ACTCCAGAATCCGCTGAAGCTCGGGATCTTAGGCCCAT-5'

For this reaction, the PCR cycle sequence was 96°C for 15 sec., 68°C for 30 sec., and 72°C for 2 minutes.

To obtain the EPOR intracellular domain including the WSXWS extracellular motif, EPOR cDNA was used with primer D (set forth above) and primer F. Primer F is the complement to primer E.

Primer F (SEQ ID NO:8)

5'-AATGGGCCTAAGATCCCGAGCTTCAGCGGATTCTGGAGT-3'



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For this reaction, the PCR cycle sequence was 96°C for 15 sec., 64°C for 30 sec., and 72°C for 2 minutes.

The second step of the two-step PCR process to generate the EECB construct was performed using the DNA fragments (agarose gel purified) from the two primary reactions in conjunction with primers A and D. The PCR cycle sequence was 96°C for 15 sec., 64°C for 30 sec., and 72°C for 2 minutes.

The hybrid receptor DNA constructs EECA and EECB were assembled using standard ligation methods into the vector pUC19 (New England Biolabs, Beverly, MA). The vectors containing the inserts were then transformed into *E. coli* cells strain DH5 alpha for amplification. The plasmids were then purified using Qiagen columns (Qiagen, Chatsworth, CA) and the inserts were subcloned into the vector pLJ (also referred to as DOL<sup>-</sup>; Korman et al., *Proc. Natl. Acad. USA*, 84:2150-2154 [1987]). This vector contains a neomycin resistance gene which affords selection for transformants using the antibiotic G418.

### 3. DNA Construct Transfection

The EECA and EECB constructs were transfected into the cultured 32D cells using the technique of electroporation. Prior to electroporation, the cells were grown to a density of about  $1 \times 10^6$  cells per ml and harvested by centrifugation at about 2,000 rpm in a clinical centrifuge for about 10 min. Next, the cells were washed twice by resuspending them in about 50 ml of electroporation medium (RPMI 1640, plus 10 ng/ml IL-3 and 10 mM HEPES buffer). After washing, the cells were resuspended in electroporation media at a density of about  $1.25 \times 10^7$  cells/ml. About 0.8 ml of the resuspended solution of cells (0.8 ml was about  $10^7$  cells) were transferred to a 4 mm electroporation cuvette (BioRad Laboratories, Richmond, CA). The cuvette was placed on ice. About 20 µg of each DNA





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construct, prepared as described above using Qiagen columns (Qiagen, Chatsworth, CA) was added to cells and mixed gently. Each cuvette of cells was transfected with one construct. The electroporations were carried out using the Biorad Gene Pulser electroporation apparatus at about 25  $\mu$ F and 1.2 kV, following the manufacturer's instructions. Immediately after electroporation, the cuvettes were placed on ice for 5-10 minutes. The cells were then gently pipetted into 100 mm Falcon petri dishes containing 25 ml of prewarmed Standard Medium. These cells were then placed in an incubator at 37° C and 5% CO<sub>2</sub> overnight. After the incubation, the cells were collected by centrifugation by spinning in a table top centrifuge at 2,000 rpm for about 10 min at room temperature. The pelleted cells were then resuspended in 25 ml of a Selective Medium which was Standard Medium plus 750  $\mu$ g/ml G418 (Geneticin, obtained from Gibco/BRL, Grand Island, NY). Approximately 1 ml of these transfected cells were plated out in each well of 24 well Falcon plates and incubated at 37° C in 5 % CO<sub>2</sub>. After 24 hours, each well was topped off with an additional 1 ml of Selective Medium, and the cells were then returned to the incubator. Those cells that had been transformed with the various DNA constructs could be identified after about one and one half to two weeks of culturing by cell colony formation in the wells. Approximately 1 in 1000 cells were transformed. All cells transformed with the same DNA were pooled and passaged in Selective Medium as described above.

#### 4. Hybrid Receptor Activity Assay

To identify those cells expressing the hybrid receptors on the plasma membrane, the cells were either analyzed by fluorescent activated cell sorting (FACS), or by growth factor selection, or by both methods.



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The cells to be sorted by FACS were prepared by washing twice in a standard solution of phosphate buffered saline supplemented with 2% fetal calf serum (Gibco/BRL, Grand Island, NY). This solution was called PBSS. The cells were incubated with a first monoclonal antibody, called Ab1, which is directed to the NH<sub>2</sub>-terminus of EGFR (Antibody Ab1; obtained from Oncogene Sciences, Manhasset, NY). Ab1 was diluted in PBSS to a concentration of 2.5 µg/ml, and 40 ml of this antibody solution was added to the cells to give a final density of about 1 X 10<sup>6</sup> cells per ml. The cells were incubated with this antibody for 1 hour at 4° C. Excess Ab1 and/or non-specific binding of Ab1 was eliminated by washing the cells twice in 50 ml of PBSS. Binding of this antibody was visualized with a second antibody directed to Ab1. This second antibody was a goat-anti-mouse IgG fluorescein isothiocyanate conjugated antibody (Southern Biotechnology Associates, Birmingham, AL). About 40 ml of this antibody at a concentration of 2.5 µg/ml was added to the cells, and the cells were incubated at about 4° C for about 1 hour. After the incubation, the cells were washed as above in PBSS. The cells were sorted using a Becton Dickinson FACS-Star Plus (San Jose, CA) following the manufacturer's guidelines. Sorting was based on the relative fluorescence of transfected cells as compared to untransfected cells. The sorted cells were allowed to recover in normal medium.

Growth factor selection of the cells was a second means used to enrich for those cells expressing the transfected hybrid receptor DNA. Both FACS sorted cells (transfected with either EGFR DNA, EECA DNA, or EECB DNA) and unsorted cells (transfected with EPOR DNA) were depleted of IL-3 by washing the cells twice in a standard solution of PBS followed by a 3 hour incubation in RPMI-1640 at 37° C. Finally, the cells were washed



- 30 -

again in PBS. After washing, the cells were seeded into 6 well cell culture plates at a concentration of about  $5 \times 10^4$  cells/ml in RPMI-1640 supplemented with 10% heat inactivated calf serum in the absence of added factors or in the presence of either 100 ng/ml IL-3, recombinant TGF-alpha at 100 ng/ml, recombinant human erythropoietin at 100  $\mu$ g/ml (Epogen® erythropoietin, Amgen Inc., Thousand Oaks, CA) or recombinant human epidermal growth factor at 100 ng/ml (EGF, Amgen Inc., Thousand Oaks, CA). Cell growth was monitored over time by sampling the cultures about every other day and counting the cells with an automated Coulter cell counter.

The activity of the hybrid receptors is shown in Figure 2. A large proportion of the cells expressing either the EPO receptor or the EGF receptor constructs were able to grow in the absence of IL-3 when EPO or EGF, respectively, were added to the growth medium. Wild-type cells and those cells transfected with control DNA only could not survive in the absence of IL-3. The EECA and EECB constructs were able to grow and survive in the absence of IL-3 when EGF was added to the culture medium.

All literature cited herein is specifically incorporated by reference.



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Amgen Inc.
- (ii) TITLE OF INVENTION: Hybrid Receptor Molecules
- (iii) NUMBER OF SEQUENCES: 8
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Amgen Inc.
  - (B) STREET: Amgen Center  
1840 Dehavilland Drive
  - (C) CITY: Thousand Oaks
  - (D) STATE: California
  - (E) COUNTRY: USA
  - (F) ZIP: 91320-1789

## (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette, 3.5 in., DS, 1.4 MB
- (B) COMPUTER: Apple Macintosh
- (C) OPERATING SYSTEM: Macintosh OS 7.0.
- (D) SOFTWARE: Microsoft Word Version 5.1

## (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION: (leave blank if not known)

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear





(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGATCAAAG TGCTGGGCTC CGGT

24

(3) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATGGTATTCT TTCTCTCCG C

21

(4) INFORMATION FOR SEQ ID NO:3 :

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCCAACGCCA CAACCACCGC GCGGGCCGC CTGACTCCG

39

(5) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TGAGAGACAG CGTCAATATT AGCGGGATCT TAGGCCCAT

40



## (6) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AATGGGCCTA AGATCCCGCT AATATTGACG CTGTCTCTCA

40

## (7) INFORMATION FOR SEQ ID NO: 6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGCAGCCACA GCTGGAAGTT AC

22

## (8) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACTCCAGAAT CCGCTGAAGC TCGGGATCTT AGGCCCAT

39



## (9) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AATGGGCCTA AGATCCCGAG CTTCAGCGGA TTCTGGAGT

39



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WE CLAIM:

1. A biologically active hybrid receptor comprising an extracellular domain and an intracellular domain, wherein one of the domains is derived from a member of the hematopoietic cytokine family of receptors, and the other domain is derived from a heterologous receptor family.
2. The hybrid receptor of claim 1 comprising EPOR extracellular domain and ANPRB intracellular domain.
3. The hybrid receptor of claim 2 further comprising ANPRB transmembrane domain.
4. The hybrid receptor of claim 1 comprising EGFR extracellular domain and EPOR intracellular domain.
5. The hybrid receptor of claim 4 further comprising EPOR transmembrane domain.
6. The hybrid receptor of claim 5 wherein EPOR intracellular domain includes the WSXWS sequence.
7. A nucleic acid sequence encoding a biologically active hybrid receptor comprising an extracellular domain and an intracellular domain, wherein one of the domains is derived from a member of the hematopoietic cytokine family of receptors, and the other domain is derived from a heterologous receptor family.
8. The nucleic acid sequence of claim 7 wherein the hybrid receptor comprises EPOR extracellular domain and ANPRB intracellular domain.





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9. The nucleic acid sequence of claim 8 wherein the hybrid receptor further comprises the ANPRB transmembrane domain. 
- 5        10. The nucleic acid sequence of claim 7 wherein the hybrid receptor comprises EGFR extracellular domain and EPOR intracellular domain.
- 10       11. The nucleic acid sequence of claim 10 wherein the hybrid receptor further comprises EPOR transmembrane domain.
- 15       12. The nucleic acid sequence of claim 11 wherein the EPOR intracellular domain includes the WSXWS sequence.
13. A vector comprising the nucleic acid sequence of claim 7.
- 20       14. A vector comprising the nucleic acid sequence of claim 8.
15. A vector comprising the nucleic acid sequence of claim 9.
- 25       16. A vector comprising the nucleic acid sequence of claim 10.
17. A vector comprising the nucleic acid sequence of claim 11.
- 30       18. A vector comprising the nucleic acid sequence of claim 12.
- 35       19. Eukaryotic cells transfected with DNA encoding the hybrid receptor of claim 1.



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20. Eukaryotic cells transfected with DNA encoding the hybrid receptor of claim 2.

21. Eukaryotic cells transfected with DNA encoding the receptor of claim 3.

22. Eukaryotic cells transfected with DNA encoding the receptor of claim 4.

23. Eukaryotic cells transfected with DNA encoding the receptor of claim 5.

24. Eukaryotic cells transfected with DNA encoding the receptor of claim 6.

25. The eukaryotic cells of claim 19 that are 32D cells.

26. The eukaryotic cells of claim 20 that are 32D cells.

27. The eukaryotic cells of claim 21 that are 32D cells.

28. The eukaryotic cells of claim 22 that are 32D cells.

29. The eukaryotic cells of claim 23 that are 32D cells.

30. The eukaryotic cells of claim 24 that are 32D cells.

31. The eukaryotic cells of claim 19 that are COS-7 cells.



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32. The eukaryotic cells of claim 20 that are COS-7 cells.

33. The eukaryotic cells of claim 21 that are COS-7  
5 cells.

34. The eukaryotic cells of claim 22 that are COS-7 cells.

10 35. The eukaryotic cells of claim 23 that are COS-7 cells.

36. The eukaryotic cells of claim 24 that are COS-7  
15 cells.

37. A method of identifying a ligand for a  
biologically active hybrid receptor comprising an  
extracellular domain and an intracellular domain,  
wherein one of the domains is derived from a member of  
20 the hematopoietic cytokine family of receptors, and the  
other domain is derived from a heterologous receptor  
family, comprising:

- (a) contacting the binding site of the hybrid  
receptor with said ligand; and
- 25 (b) detecting a cellular response to the  
ligand.



23. An antibody immunospecific for the polypeptide or immunological fragment as claimed in any one of claims 1 to 5.

24. A method of diagnosing a *Neisseria meningitidis* infection, comprising identifying a polypeptide as claimed in any one of claims 1 - 5, or an antibody that is immunospecific for said polypeptide, present within a biological sample from an animal suspected of having such an infection.

25. Use of a composition comprising an immunologically effective amount of a polypeptide as claimed in any one of claims 1 - 5 in the preparation of a medicament for use in generating an immune response in an animal.

26. Use of a composition comprising an immunologically effective amount of a polynucleotide as claimed in any one of claims 6 - 15 in the preparation of a medicament for use in generating an immune response in an animal.

27. A therapeutic composition useful in treating humans with *Neisseria meningitidis* disease comprising at least one antibody directed against the polypeptide of claims 1 - 5 and a suitable pharmaceutical carrier.

20

16. An expression vector or a recombinant live microorganism comprising an isolated polynucleotide according to any one of claims 6 - 15.

17. A host cell comprising the expression vector of claim 16 or a subcellular fraction or a membrane of said host cell expressing an isolated polypeptide comprising an amino acid sequence that has at least 85% identity to the amino acid sequence selected from the group consisting of: SEQ ID NO:4 or SEQ ID NO:6.

18. A process for producing a polypeptide comprising an amino acid sequence that has at least 85% identity to the amino acid sequence selected from the group consisting of SEQ ID NO:4 or SEQ ID NO:6 comprising culturing a host cell of claim 17 under conditions sufficient for the production of said polypeptide and recovering the polypeptide from the culture medium.

19. A process for expressing a polynucleotide of any one of claims 6 - 15 comprising transforming a host cell with the expression vector comprising at least one of said polynucleotides and culturing said host cell under conditions sufficient for expression of any one of said polynucleotides.

20. A vaccine composition comprising an effective amount of the polypeptide of any one of claims 1 to 5 and a pharmaceutically acceptable carrier.

21. A vaccine composition comprising an effective amount of the polynucleotide of any one of claims 6 to 15 and a pharmaceutically acceptable carrier.

22. The vaccine composition according to either one of claims 20 or 21 wherein said composition comprises at least one other *Neisseria meningitidis* antigen.



8. An isolated polynucleotide which comprises a nucleotide sequence which has at least 85% identity to that of SEQ ID NO: 3 or 5 over the entire length of SEQ ID NO: 3 or 5 respectively; or a nucleotide sequence complementary to said isolated polynucleotide.
- 5 9. The isolated polynucleotide as claimed in any one of claims 6 to 8 in which the identity is at least 95% to SEQ ID NO: 3 or 5.
10. An isolated polynucleotide comprising a nucleotide sequence encoding the polypeptide of SEQ ID NO:4 or SEQ ID NO:6.
- 10 11. An isolated polynucleotide comprising the polynucleotide of SEQ ID NO:3 or SEQ ID NO:5.
12. An isolated polynucleotide comprising a nucleotide sequence encoding the polypeptide  
15 of SEQ ID NO:4 or SEQ ID NO:6, obtainable by screening an appropriate library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO:3 or SEQ ID NO:5 or a fragment thereof.
13. An isolated polynucleotide comprising a nucleotide sequence encoding the polypeptide  
20 of SEQ ID NO:2.
14. An isolated polynucleotide comprising the polynucleotide of SEQ ID NO:1.
15. An isolated polynucleotide comprising a nucleotide sequence encoding the polypeptide  
25 of SEQ ID NO:2, obtainable by screening an appropriate library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO:1 or a fragment thereof.

## CLAIMS


1. An isolated polypeptide comprising an amino acid sequence which has at least 85% identity to the amino acid sequence selected from the group consisting of: SEQ ID NO:2,  
5 SEQ ID NO:4 and SEQ ID NO:6.
2. An isolated polypeptide as claimed in claim 1 in which the amino acid sequence has at least 95% identity to the amino acid sequence selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.  
10
3. The polypeptide as claimed in claim 1 comprising the amino acid sequence selected from the group consisting of: SEQ ID NO: 2, SEQ ID NO:4 and SEQ ID NO:6.
4. An isolated polypeptide of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.  
15
5. An immunogenic fragment of the polypeptide as claimed in any one of claims 1 to 4 in which the immunogenic activity of said immunogenic fragment is substantially the same as the polypeptide of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.
- 20 6. An isolated polynucleotide comprising a nucleotide sequence encoding a polypeptide that has at least 85% identity to the amino acid sequence of SEQ ID NO: 4 or 6 over the entire length of SEQ ID NO: 4 or 6 respectively; or a nucleotide sequence complementary to said isolated polynucleotide.
- 25 7. An isolated polynucleotide comprising a nucleotide sequence that has at least 85% identity to a nucleotide sequence encoding a polypeptide of SEQ ID NO: 4 or 6 over the entire coding region; or a nucleotide sequence complementary to said isolated polynucleotide.

Applicant's or agent's file reference	KLP/BM45339	International application No.
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**INDICATIONS RELATING TO DEPOSITED MICROORGANISM  
OR OTHER BIOLOGICAL MATERIAL**

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>63</u> , line <u>3-22</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution  AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 10801 UNIVERSITY BLVD, MANASSAS, VIRGINIA 20110-2209, UNITED STATES OF AMERICA	
Date of deposit 22 June 1997 (22.06.97)	Accession Number 13090
C. ADDITIONAL INDICATIONS (leave blank if not applicable)	
This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations where a European Patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European Patent or until the date on which the application has been refused or withdrawn, only by issue of such a sample to an expert nominated by the person requesting the sample.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer 	Authorized officer

**Deposited materials**

A deposit containing a *Neisseria meningitidis* Serogroup B strain has been deposited with the American Type Culture Collection (herein "ATCC") on June 22, 1997 and assigned  
5 deposit number 13090. The deposit was described as *Neisseria meningitidis* (Albrecht and Ghon) and is a freeze-dried, 1.5-2.9 kb insert library constructed from *N. meningitidis* isolate. The deposit is described in Int. Bull. Bacteriol. Nomencl. Taxon. 8: 1-15 (1958).

The *Neisseria meningitidis* strain deposit is referred to herein as "the deposited strain" or as  
10 "the DNA of the deposited strain."

The deposited strain contains the full length BASB040 gene. The sequence of the polynucleotides contained in the deposited strain, as well as the amino acid sequence of any polypeptide encoded thereby, are controlling in the event of any conflict with any  
15 description of sequences herein.

The deposit of the deposited strain has been made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for Purposes of Patent Procedure. The strain will be irrevocably and without restriction or condition released to the  
20 public upon the issuance of a patent. The deposited strain is provided merely as convenience to those of skill in the art and is not an admission that a deposit is required for enablement, such as that required under 35 U.S.C. §112.

AADLSENKAAGFALFKNKSPDTESVKLKPKFPVLIDTQDSEIKDMVEEHLPLITQQQEEVLDKEQTGFLAEEAPDNVKTMLRSKGYFSSKVSLETEKD GAYTVHITPGPRTKIANVGVAI LGDILSDGNLA EYYRNALENWQQPVGSDFDQDSWENSKTSV LGAVTRKAYPLAKLGNTQAAVNPD TATADLNVVDSGRPIAFGD FEITGTQRYPEQIVSGLARFQPGMPYDL D L L L D F Q Q ALEQNGHYS GASVQADFRLQGD RVPVKVSVTEVKRHKLETGIRLDSEYGLGGKIA YDYYNLFNKGYIGSVVWMDKYET

5 TLAAGISQPRNYRGNYWTSNVSYNRSTTQNLKRAFSGGVVYVRDRAGIDARLGAEFLA EGRKIPGSAVDLGN SHATMLT ASWKRQLLNNVLHPENGHYLDGKIGTTLGTFLSSTALIRTSARAGYFFT PENKKLGTFIIRGQAGYTVARDNADVP SGLM FRSGGASSVRGYELDSIGLAGPNGSVLPERALLVGSLEYQLPFTRTL SGAVFHDMDAAANFKRMKLKHGSGLGVRWFSP LAPFSFDIAYGHSDKKIRWHISLGTRF

10 SEQ ID NO:7  
GGG CCG CAA CCT CCG AAA TA

SEQ ID NO:8  
CGA GCC AGC CGA GGA AAC ATA

15 SEQ ID NO:9  
CAT AGC ACC ATG GCC GCC GAC CTT TCC GA

20 SEQ ID NO:10  
CTA GTC TAG ATT AGA AGC GTG TTC CCA AGC

SEQ ID NO:11  
*Neisseria meningitidis* polynucleotide sequence up-stream of the BASB040 gene sequence, in strain ATCC 13090

25 AAACGGATAATCCATTTACGACGGTGGAAACCGCTTCGCCAAATCGGCAACGACCTGCCGCGTAACGTCCGAACCGTG  
ATTCGCAACGCGCGCCCAAAACCAAGCCCAAGCCAAATGCCGATATAGTTGGCATTGGCAATCGCGTTAATCGGGT  
TGGCGACCAGGTT CATCAGCAGCGATTTCACACTTCCACAATGCCGAAGGCGGCGCGGACACATCGCCCGCGCCC  
GCCAAAACAATGTGCGTCGGGAAAACCATACCGGCGATGACGGCGGTGAGGGCTGCGGAAAACGTACCAATGAGGTAAG  
30 GATGATAATCGGCCTGATATGCGCCTTGTTGCTTTTGGTGCTGCGCGATTGTGGCCGCCACCAAAATAAATACCAAAA  
CCGGCGCGACCGCTTTGAGCGCGCGACAAAACAGGCTGCCGAACAAGCCTGCCGCCAAGCCCAGTTGCGGGGAAACCGAA  
CCGATTACGATGCCCAACGCCAAACCGGCGGCAATCGCCTGACCAGGCTGACGCGGCCGATCGCATGAAATAAGGATTT  
GCCGAACGCCATAATTCTTCCTTATGTTGTGATATGTTAAAAATGTTGTATTTTAAAGAAAACCTATTCTCTGTGTTT  
TTTTTATTTTCGGCTGTGTTTTAAGGTGCGTTGATTGCCCCTATGCAGTGCCGGACAGGCTTTGCTTTATCATTCCGGC  
35 GCAACGGTTTAATTTATTGAACGAAAATAAATTTATTTAATCCTGCCTATTTCCGGCACTATTCGAAACGCAGCCTGT  
TTTCCATATGCGGATTGGAAACAAAATACCTTAAAAACAAGCAGATACATTTCCGGCGGGCCGCAACCTCCGAAATACCGG  
CGGCAGTATGCCGTCTGAAGTGTCCCGCCCCGTCCGAACAACAAAAACAGCCGTTCGAAACCTGTCCGAACAGTGTT  
AGAATCGAAATCTGCCACACCGATGCACGACACCCGTACC

EVLDKEQTGFLAEEAPDNVKTMLRSKGYFSSKVSLETKDAYTVHITPGPRTKIANVGVAILGDI LSDGNLA EYYRNALE  
NWQQPVGSDFDQDSWENSKTSVLGAVTRKGYPLAKLGNTAAVNPDATVDLNVVVD SGRPIAFGD FEITGTQRYPEQIV  
SGLARFQPGTPYDL DLLDFQQA LEQNGHYSGASVQADFDRLQGD RVPVKVSVTEVKRHKLETGIRLDSEYGLGGKIAYD  
YYNLFNKGYIGSVVWMDKYETTLAAGISQPRNYRGNWTSNVSYNRSTTQNLKRAFSGGIWYVRDRAGIDARLGAEFL  
5 AEGRKIPGSDIDLGN SHATMLTASWK RQLLNVLHPENGHYLDGKIGITTLGTFLSSTALIRTSARAGYFFT PENKKLGTF  
IIRGOAGYTVARDNADVP SGLMFRSGGASSVRGYELDSIGLAGPNGSVLPERALLVGSLEYQLPFTRTL SGAVFHD MGDA  
AANFKRMKLKHGSGLGVRWFSPLAPFSFDIAYGHSDKKIRWHISLGTRF

## 10 SEQ ID NO:5

*Neisseria meningitidis* BASB040 polynucleotide sequence from strain H44/76

GCCGCCGACCTTTCCGAAAACAAGGCGGCGGGTTTCGCATTGTTCAAAAACAAAAGCCCCGACACCGAATCAGTCAAATT  
AAAACCCAAATCCCCGTCTCATCGACACGCAGGACAGTGAAATCAAAGATATGGTCGAAGAACACCTGCCGCTCATCA  
15 CGCAGCAGCAGGAAGAAGTATTGGACAAGGAACAGACGGGCTTCTCGCGAAGAAGCGCCGGACAACGTTAAACGATG  
CTCCGCAGCAAAGGCTATTTAGCAGCAAAGTCAGCCTGACGAAAAAGACGGAGCTTATACGGTACACATCACACCGGG  
CCCGCGCACAAAATCGCCAACGTGGCGCTCGCCATCCTCGGCGACATCCTTTCAGACGGCAACCTCGCCGAATACTACC  
GCAACGCGCTGGAAAACTGGCAGCAGCCGCTAGGCAGCGATTTGATCAGGACAGTTGGGAAAAACAGCAAACTTCCGTC  
CTCGGCGCGGTAACGCGCAAAGCCTACCCGCTTGCCAAGCTCGGCAATACGACGCGGCCGTCACCCCGATACCGCCAC  
20 CGCCGATTGTAACGTCGTCGTGGACAGCGCGCCGCCATCGCCTTCGGCGACTTTGAAATCACCGGCACACAGCGTTACC  
CCGAACAAATCGTCTCGGCCTTGCGGCTTTCAGCCCGGTATGCCGTACGACCTCGACCTGCTGCTCGACTTCCAACAG  
GCGCTCGAACAAAACGGGCATTATTCGGCGCGTCCGTACAAGCCGACTTCGACCGCCTCCAAGGCGACCGCGTCCCGT  
CAAAGTCAGCGTAACCGAGGTCAAACGCCACAACTCGAAACCGGCATCCGCCTCGATTCCGAATACGGTTTGGGCGGCA  
AAATCGCCTACGACTATTACAACCTCTTCAACAAAGGCTATATCGGTTTCGGTCGTCTGGGATATGGACAAATACGAAACC  
25 ACGCTTGGCCCGGCATCAGCCAGCCGCGCAACTATCGGGGCAACTACTGGACAAGCAACGTTTCTTACAACCGTTTCGAC  
CACCCAAAACCTCGAAAAACCGGCCTTCTCGGCGGCGTCTGGTATGTGCGCGACCGCGGGCATCGATGCCAGGCTGG  
GGGCGGAATTTCTCGCAGAAGGCCGAAAAATCCCGGCTCGGCTGTGATTTGGGCAACAGCCACGCCACGATGCTGACC  
GCCTCTTGGAACGCCAGCTGCTCAACAACGTGTGCATCCCGAAAACGGCCATTACCTCGACGGCAAAATCGGTACGAC  
TTTGGGCACATTCTGTCTCTCCACCGCGTGATCCGCACCTCTGCCCGTGACGTTATTTCTTACGCCCGAAAAACAAA  
30 AACTCGGCACGTTTCATACGCGGACAAGCGGGTTACACCGTTGCCCGGACAATGCCGACGTTCTTTCAGGGCTGATG  
TTCCGCAGCGGCGCGCGTCTTCCGTGCGCGGTTACGAACTCGACAGCATCGGACTTGCCGGCCCGAACGGATCGGTCTT  
GCCCGAACGCGCCCTCTGGTGGGCGCCTGGAATACCACTGCCGTTTACGCGCACCTTTCCGGCGGGTGTTCCAGG  
ATATGGGCGATGCCGCCGCAATTTCAAACGTATGAAGCTGAAACACGGTTCCGGACTGGGCGTGCGCTGGTTACGCCG  
CTTGCGCGGTTTTCTTTCGACATCGCCTACGGGCACAGCGATAAGAAAAATCCGCTGGCACATCAGCTTGGGAACAGCCTT  
35 CTAA

## SEQ ID NO:6

*Neisseria meningitidis* BASB040 polypeptide sequence deduced from the polynucleotide of  
SeQ ID NO:5

40

SGLARFQPGTPYDLDDLDFQQALEQNGHYSVASVQADFRLQGDVPVKVSVTEVKRHK  
 LETGIRLDSEYGLGGKIAYDYNNLFNKGYIGSVVWMDKYETTLAAGISQPRNYRGNWYWT  
 SNVSYNRSTTQNLKRAFSGGIWYVRDRAGIDARLGAFLAEGRKIPGSDIDLGNSHATM  
 LTASWKRQLLNNVLHPENGHYLDGKIGTTLGTFLSSTALIRTSARAGYFFT PENKKLGTF  
 5 IIRGQAGYTVARDNADVPSGLMFRSGGASSVRGYELDSIGLAGPNGSVLPERALLVGSLE  
 YQLPFTRTLSGAVFHDMDAAANFKRMKLGSGLGVRWFSPLAPFSFDIAYGHSDKKIR  
 WHISLGTRF

### SEQ ID NO:3

10 *Neisseria meningitidis* BASB040 polynucleotide sequence from strain ATCC 13090

ATGATGATCAAACCGACCGCCCTGCTCCTGCGGCTTATTTTCTTCCGCACGCATACGCGCCTGCCGCGACCTTTC  
 CGAAAAACAAGGCGGCGGGTTTCGATTGTTCAAAAACAAAAGCCCCGACACCGAATCAGTTAAATTAAACCCAAATTCC  
 CCGTCCGCATCGACACGCAGGATAGTGAAATCAAAGATATGGTCGAAGAACACCTGCCGCTCATCACGCAGCAGCAGGAA  
 15 GAAGTATTGGACAAGGAACAGACGGGCTTCTCGCCGAAGAAGCACCGGACAACTTAAACCATGCTCCGCAGCAAAGG  
 CTATTTTCAGCAGCAAAGTCAGCCTGACGGAAAAAGACGGAGCTTATACGGTACACATCACACCGGGCCCGCGCACCAAAA  
 TCGCCAACGTCCGTGTGCGCATCCTCGGCGACATCCTTTCAGACGGCAACCTCGCCGAATACTACCGCAACGCGCTGGAA  
 AACTGGCAGCAGCCGGTAGGCAGTGATTTTCGATCAGGACAGTTGGGAAAAACAGCAAACCTTCCGTCTCGGCGGGTAAC  
 GCGCAAAGGCTACCGCTTGCCAAGCTCGGCAACACCCGGGCGGCCGTCAACCCCGATACCGCCACCGTTCGATTGTAACG  
 20 TCGTCGTGGACAGCGGCGCCCATCGCCTTCGGCGACTTTGAAATCACCGGCACACAGCGTTACCCCGAACAAATCGTC  
 TCCGGCCTGGCGCGCTTCAAACCGGGCACGCCCTACGACCTCGACCTGCTGCTCGACTTCCAACAGGCACCTCGAACAAAA  
 CGGGCATTATTCGGGCGCGTCCGTACAAGCCGACTTCGACCGTCTCCAAGGCGACCGGTCCCGCTCAAAGTCAGCGTAA  
 CCGAGGTCAAACGCCACAAGCTCGAAACCGGCATCCGCTCGATTTCGAATACGGTTTGGGCGGCAAAATCGCCTACGAC  
 TATTACAACCTCTTCAACAAAGGCTATATCGGCTCGGTCTGCGGATATGGACAAATACGAAACACGCTTGCCGCGGG  
 25 CATCAGCCAGCCGCGCAACTATCGGGGCAACTACTGGACAAGCAACGTTTCTTACAACCGTTTCGACCACCCAAAACCTCG  
 AAAACGCGCCTTCTCCGGCGGCATCTGGTATGTGCGCGACCGCGCGGCATCGATGCCAGGCTGGGGGAGAGTTTCTC  
 GCAGAAGGCCGGAAAAATCCCGGCTCGGATATCGATTTGGGCAACAGCCACGCCAGATGCTGACCGCTCTTGAAACG  
 CCAGCTGCTCAACAACGTGCTGCATCCCGAAAAACGGCCATTACCTCGACGGCAAAATCGGTACGACTTTGGGCACATTCC  
 TGTCCTCCACCGCGCTGATCCGCACCTCTGCCCGTGCGAGTTATTTCTTACGCCCGAAAAACAAAAACTCGGCACGTTTC  
 30 ATCATACGCGGACAAGCGGGTTACACCGTTGCCCGGACAATGCCGACGTTCTTTCAGGGCTGATGTTCCGCAGCGCGG  
 CGCGTCTTCCGTGCGCGGTACGAACTCGACAGCATCGGACTTGCCGGCCCGAACGGATCGGTCTGCCCCGAACGCGGCC  
 TCCTGGTGGGCAGCCTGGAATACCAACTGCCGTTTACGCGCACCTTTCCGGCGCGGTGTTCCACGATATGGGCGATGCC  
 GCCGCCAATTTCAAACGTATGAAGCTGAAACACGGTTTCGGGACTGGGCGTGCGTGGTTAGCCCCGCTTGCGCCGTTTTTC  
 CTTTCGACATCGCCTACGGGCACAGCGATAAGAAAATCCGCTGGCACATCAGCTTGGGAACACGCTTCTAA

35

### SEQ ID NO:4

*Neisseria meningitidis* BASB040 polypeptide sequence deduced from the polynucleotide of  
SeQ ID NO:3

40 MMIKPTALLLPALFFPHAYAPAADLSENKAAGFALFKNKSPTESVKLPKFPVRIDTQDSEIKDMVEEHLPLITQQQE

## SEQUENCE INFORMATION

## BASB040 Polynucleotide and Polypeptide Sequences

## SEQ ID NO:1

5 *Neisseria meningitidis* BASB040 polynucleotide sequence from strain ATCC 13090

ATGATGATCAAACCGACCGCCCTGCTCCTGCCGGCTTTATTTTCTTTCCGCACGCATAC  
 GCGCTGCGCCGACCTTTCCGAAAACAAGGCGGCGGGTTTCGCATTGTTCAAAAAACAA  
 AGCCCCGACACCGAATCAGTTAAATTAAAACCCAAATCCCCGTCCGCATCGACACGCAG  
 10 GATAGTGAAATCAAAGATATGGTCGAAGAACACCTGCCGCTCATCACGCAGCAGCAGGAA  
 GAAGTATTGGACAAGGAACAGACGGGCTTCCTCGCCGAAGAAGCACCGGACAACGTTAAA  
 ACCATGCTCCGCAGCAAAGGCTATTTTCAGCAGCAAAGTCAGCCTGACGGAAAAAGACGGA  
 GCTTATACGGTACACATCACACCGGGCCCCGCGCACAAAATCGCCAACGTCGGTGTGCGC  
 ATCCTCGGCGACATCCTTTCAGACGGCAACCTCGCCGAATACTACCGCAACGCGCTGGAA  
 15 AACTGGCAGCAGCCGGTAGGCAGTGATTTTCGATCAGGACAGTTGGGAAAACAGCAAACT  
 TCCGTCTCGGCGCGGTAACGCGAAAAGGCTACCCGCTTGCCAAGCTCGGCAACACCCGG  
 GCGGCGGTCAACCCCGATACCGCCACCGTCGATTGTAACGTCGTCTGGACAGCGGCGCG  
 CCCATCGCCTTCGGCGACTTTGAAATCACCGGCACACAGCGTTACCCGAACAAATCGTC  
 TCCGGCCTGGGCGGCTTCCAACCGGGCACGCCCTACGACCTCGACCTGCTGCTCGACTTC  
 20 CAACAGGCACTCGAACAAAACGGGCATTATTCGGGCGGTCCGTACAAGCCGACTTCGAC  
 CGTCTCCAAGGCGACCGCGTCCCCGTCAAAGTCAGCGTAACCGAGGTCAAACGCCACAAG  
 CTCGAAACCGGCATCCGCCTCGATTGGAATACGGTTTGGGCGGCAAAATCGCCTACGAC  
 TATTACAACCTCTTCAACAAAGGCTATATCGGCTCGGTCTGCTGGGATATGGACAAATAC  
 GAAACCACGCTTGCCGCCGGCATCAGCCAGCCGCGCAACTATCGGGGCAACTACTGGACA  
 25 AGCAACGTTTCTTACAACCGTTTCGACCACCCAAAACCTCGAAAAACGCGCCTTCTCCGGC  
 GGCATCTGGTATGTGCGCGACCGCGCGGGCATCGATGCCAGGCTGGGGGCAGAGTTTCTC  
 GCAGAAGGCCGGAAAAATCCCCGGCTCGGATATCGATTGTTGGGCAACAGCCACGCCACGATG  
 CTGACCGCCTCTTGGAAACGCCAGCTGCTCAACAACGTGCTGCATCCGAAAACGGCCAT  
 TACCTCGACGGCAAAATCGGTACGACTTTGGGCACATTCTGTCTCCACCGCGCTGATC  
 30 CGCACCTCTGCCCGTCAGGTTATTTCTTCACGCCCCGAAAACAAAAAACTCGGCACGTTT  
 ATCATACGCGGACAAGCGGGTTACACCGTTGCCCGCGACAATGCCGACGTTCTTTCAGGG  
 CTGATGTTCCGCAGCGGCGGCGCGTCTTCCGTGCGCGGTTACGAACTCGACAGCATCGGA  
 CTTGCCGGCCCCGAACGGATCGGTCTGCCCCGAACGCGCCCTCCTGGTGGGCGAGCCTGGAA  
 TACCAACTGCCGTTTACGCGCACCTTTCCGGCGCGGTGTTCCACGATATGGGCGATGCC  
 35 GCCGCCAATTTCAAACGTATGAAGCTGAAACACGGTTTCGGGACTGGGCGTGCCTGGTTC  
 AGCCCCGTTGCGCCGTTTTCTTTCGACATCGCCTACGGGCACAGCGATAAGAAAAATCCGC  
 TGGCACATCAGCTTGGGAACACGCTTCTAA

## SEQ ID NO:2

40 *Neisseria meningitidis* BASB040 polypeptide sequence deduced from the polynucleotide of  
 SeQ ID NO:1

MMIKPTALLLPALFFPHAYAPAADLSENKAAGFALFKNKSPDTESVKLKPKFPVRIDTQ  
 DSEIKDMVEEHLPLITQQQEEVLDKEQTGFLAEEAPDNVKTMLRSKGYFSSKVSLETKDG  
 45 AYTIVHITPGPRTKIANVGVAILGDI LSDGNLAEYYRNALENWQQPVGSDFDQDSWENSKT  
 SVLGAVTRKGYPLAKLGNTRAAVNPDATVDLNVVVD SGRPIAFGDFEITGTQRYPEQIV



homologous recombination). Upregulated expression can be obtained in both the bacterium as well as in the outer membrane vesicles shed (or made) from the bacterium.

5 In other examples, the described approaches can be used to generate recombinant bacterial strains with improved characteristics for vaccine applications. These can be, but are not limited to, attenuated strains, strains with increased expression of selected antigens, strains with knock-outs (or decreased expression) of genes interfering with the immune response, strains with modulated expression of immunodominant proteins, strains with modulated shedding of outer-membrane vesicles.

10

A region directly upstream of the BASB040 gene is given in the sequence of SEQ ID NO:11. This sequence is a further aspect of the invention.

by similar types of modifications. Alternatively, by changing phase variation sequences, the expression of the gene can be put under phase variation control, or may be uncoupled from this regulation. In another approach, the expression of the gene can be put under the control of one or more inducible elements allowing regulated expression.

- 5 Examples of such regulation include, but are not limited to, induction by temperature shift, addition of inductor substrates like selected carbohydrates or their derivatives, trace elements, vitamins, co-factors, metal ions, etc.

Such modifications as described above can be introduced by several different means.

- 10 The modification of sequences involved in gene expression can be done *in vivo* by random mutagenesis followed by selection for the desired phenotype. Another approach consists in isolating the region of interest and modifying it by random mutagenesis, or site-directed mutagenesis, insertion or deletion mutagenesis. The modified region can then be reintroduced into the bacterial genome by homologous recombination, and the
- 15 effect on gene expression can be assessed. In another approach, the sequence knowledge of the region of interest can be used to replace or delete all or part of the natural regulatory sequences. In this case, the regulatory region targeted is isolated and modified so as to contain the regulatory elements from another gene, a combination of regulatory elements from different genes, a synthetic regulatory region, or any other
- 20 regulatory region, or to delete selected parts of the wild-type regulatory sequences. These modified sequences can then be reintroduced into the bacterium via homologous recombination into the genome. A non-exhaustive list of preferred promoters that could be used for up-regulation of gene expression includes the promoter *porA*, *porB*, *lbpB*, *tbpB*, *pI10*, *lst*, *hpuAB* from *N. meningitidis* or *N. gonorrhoeae*.

25

In one example, the expression of the gene can be modulated by exchanging its promoter with a stronger promoter (through isolating the upstream sequence of the gene, *in vitro* modification of this sequence, and reintroduction into the genome by

at room temperature for ~3 minutes. Individual cell pellets were suspended in ~50µl of sterile water, then mixed with an equal volume of 2X Laemelli SDS-PAGE sample buffer containing 2-mercaptoethanol, and placed in boiling water bath for ~3 min to denature protein. Equal volumes (~15µl) of both the crude arabinose-induced and the non-induced cell lysates were loaded onto duplicate 12% Tris/glycine polyacrylamide gel (1 mm thick Mini-gels, Novex). The induced and non-induced lysate samples were electrophoresed together with prestained molecular weight markers under conventional conditions using a standard SDS/Tris/glycine running buffer. Following electrophoresis, one gel was stained with commassie brilliant blue R250 (BioRad) and then destained to visualize novel BASB040 arabinose-inducible protein(s).

**Example 3 : Analysis of the non-coding flanking regions of the BASB040 gene, and its exploitation for modulated BASB040 gene expression.**

The non-coding flanking regions of the BASB040 gene contain regulatory elements important in the expression of the gene. This regulation takes place both at the transcriptional and translational level. The sequence of these regions, either upstream or downstream of the open reading frame of the gene, can be obtained by DNA sequencing. This sequence information allows the determination of potential regulatory motifs such as the different promoter elements, terminator sequences, inducible sequence elements, repressors, elements responsible for phase variation, the shine-dalgarno sequence, regions with potential secondary structure involved in regulation, as well as other types of regulatory motifs or sequences.

This sequence information allows the modulation of the natural expression of gene BASB040. The upregulation of the gene expression may be accomplished by altering the promoter, the shine-dalgarno sequence, potential repressor or operator elements, or any other elements involved. Likewise, downregulation of expression can be achieved

for cloning, purified PCR product was sequentially digested to completion with *Nco*I and *Xba*I restriction enzymes as recommended by the manufacturer (Boehringer Mannheim). Digested BASB040 PCR products and pBAD were gel-purified and ligated together using an approximately 5-fold molar excess of the digested fragment to the  
5 vector. A standard ~20 µl ligation reaction (~16°C, ~16 hours), using methods well known in the art, was performed using T4 DNA ligase (~2.0 units / reaction, Boehringer Mannheim). An aliquot of the ligation was used to transform electro-competent *E. coli* Top10 cells according to methods well known in the art. Following a ~2-3 hour outgrowth period at 37°C in ~1.0 ml of LB broth, transformed cells were plated on LB  
10 agar plates containing Ampicillin (50 µg/ml). Individual ampicillin-resistant colonies were selected and analyzed by whole cell-based PCR to verify that transformants contained the BASB040 DNA insert. Transformants that produced the expected PCR product were identified as strains containing a BASB040 expression construct. Expression plasmid containing strains were then analyzed for the inducible expression  
15 of recombinant BASB040.

#### B: Expression Analysis of PCR-Positive Transformants.

For each PCR-positive transformant identified above, ~5.0 ml of LB broth containing ampicillin (50 µg/ml) was inoculated with cells from the patch plate and grown  
20 overnight at 37 °C with shaking (~250 rpm). An aliquot of the overnight seed culture (~1.0 ml) was inoculated into a 125 ml erlenmeyer flask containing ~25 ml of LB Kn broth and grown at 37 °C with shaking (~250 rpm) until the culture turbidity reached O.D.600 of ~0.5, i.e. mid-log phase (usually about 1.5 - 2.0 hours). At this time approximately half of the culture (~12.5 ml) was transferred to a second 125 ml flask  
25 and expression of recombinant BASB040 protein induced by the addition of L-Arabinose to a final concentration of 0.2 % (w/v). Incubation of both the arabinose-induced and non-induced cultures continued for an additional ~4 hours at 37 °C with shaking. Samples (~1.0 ml) of both induced and non-induced cultures were removed after the induction period and the cells collected by centrifugation in a microcentrifuge

**Table 1: Pairwise identities of the BASB040 polynucleotide sequences ( in % )**

	SeqID No:3	SeqID No:5
SeqID No:1	99.9	98.3
SeqID No:3		98.4

5 **Table 2: Pairwise identities of the BASB040 polypeptide sequences ( in % )**

	SeqID No:4	SeqID No:6
SeqID No:2	100.	99.0
SeqID No:4		99.0

**Example 2: Construction of Plasmid to Express Recombinant BASB040**

10

**A: Cloning of BASB040.**

The *Nco*I and *Xba*I restriction sites (underlined) engineered into the forward D15b-01 (5'- CAT AGC ACC ATG GCC GCC GAC CTT TCC GA -3') [SEQ ID NO:9] and reverse D15b-02 ( 5'- CTA GTC TAG ATT AGA AGC GTG TTC CCA AGC -3') [SEQ ID NO:10] amplification primers, respectively, permitted directional cloning of a BASB040 PCR product into the commercially available *E. coli* expression plasmid pBADgIII(A) (Invitrogen, USA, ampicillin resistant). This plasmid provides the signal peptide from the bacteriophage fd pIII protein such that a mature BASB040 protein could be targeted to the periplasm of *E. coli*. The BASB040 PCR product was purified from the amplification reaction using Wizard PCR prep<sup>TM</sup> (Promega) according to the manufacturers instructions. To produce the required *Nco*I and *Xba*I termini necessary

20

The sequence of the BASB040 gene was also determined in another *N. meningitidis* serogroup B strain, the strain H44/76. For this purpose, plasmid DNA (see example 2A) containing the gene region encoding the mature BASB040 from *N. meningitidis* strain H44/76 was used as a PCR template. This material (1µg) was then submitted to

5 Polymerase Chain Reaction DNA amplification using primers D15b-01 (5'- CAT AGC ACC ATG GCC GCC GAC CTT TCC GA -3') [SEQ ID NO:9] and D15b-02 (5'- CTA GTC TAG ATT AGA AGC GTG TTC CCA AGC -3') [SEQ ID NO:10] specific for the BASB040 gene. The PCR amplicon was then submitted to DNA sequencing using the Big Dyes kit (Applied biosystems) and analyzed on a ABI 373/A DNA

10 sequencer in the conditions described by the supplier. As a result, the polynucleotide and deduced polypeptide sequences, referred to as SEQ ID NO:5 and SEQ ID NO:6 respectively, were obtained.

Using the MegAlign program from the DNASTAR software package, an alignment of

15 the polynucleotide sequences of SEQ ID NO:1, 3 and 5 was performed, and is displayed in Figure 1; a pairwise comparison of identities is summarized in Table 1, showing that the three BASB040 polynucleotide gene sequences are all similar at identity level greater than 98.0 %. Using the same MegAlign program, an alignment of the polypeptide sequences of SEQ ID NO:2, 4 and 6 was performed, and is displayed in

20 Figure 2; a pairwise comparison of identities is summarized in Table 2, showing that the three BASB040 protein sequences are all similar at an identity level greater than or equal to 99.0 %.

Taken together, these data indicate strong sequence conservation of the BASB040 gene among the two *N.meningitidis* serogroup B strains.

25

**EXAMPLES:**

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples  
5 are illustrative, but do not limit the invention.

**Example 1: Discovery and confirmatory DNA sequencing of the BASB040 gene from two *N.meningitidis* strains.**

10 **A: BASB040 in *N. meningitidis* serogroup B strain ATCC13090.**

The BASB040 gene of SEQ ID NO:1 was first discovered in the Incyte PathoSeq database containing unfinished genomic DNA sequences of the *N. meningitidis* strain ATCC13090. The translation of the BASB040 polynucleotide sequence, shown in SEQ ID NO:2, showed significant similarity ( 21 % identity in a 623 amino acids overlap) to  
15 the *Neisseria meningitidis* D15 protective surface.

The sequence of the BASB040 gene was further confirmed experimentally. For this purpose, genomic DNA was extracted from  $10^{10}$  cells of the *N.meningitidis* cells (strain ATCC 13090) using the QIAGEN genomic DNA extraction kit (Qiagen GmbH), and 1 µg of this material was submitted to Polymerase Chain Reaction DNA amplification  
20 using primers D15b-21-ctg (5'-GGG CCG CAA CCT CCG AAA TA-3') [SEQ ID NO:7] and D15b-22-ctg (5'-CGA GCC AGC CGA GGA AAC ATA C -3') [SEQ ID NO:8]. This PCR product was gel-purified and subjected to DNA sequencing using the Big Dye Cycle Sequencing kit (Perkin-Elmer) and an ABI 373A/PRISM DNA sequencer. DNA sequencing was performed on both strands with a redundancy of 2 and  
25 the full-length sequence was assembled using the SeqMan program from the DNASTAR Lasergene software package. The resulting DNA sequence and deduced polypeptide sequence are shown as SEQ ID NO:3 and SEQ ID NO:4 respectively.

**B: BASB040 in *N. meningitidis* serogroup B strain H44/76.**

"Polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA including single and double-stranded regions.

- 5 "Variant" refers to a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes  
10 may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical.
- 15 A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring  
20 variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

- "Disease(s)" means any disease caused by or related to infection by a bacteria, including , for example, upper respiratory tract infection, invasive bacterial diseases, such as bacteremia  
25 and meningitis.



substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence. The number of amino acid alterations for a given % identity is determined by multiplying the total number of amino acids in SEQ ID NO:2 by the integer defining the percent identity divided by 100 and then subtracting that product from said total number of amino acids in SEQ ID NO:2, or:

$$n_a \leq x_a - (x_a \bullet y),$$

wherein  $n_a$  is the number of amino acid alterations,  $x_a$  is the total number of amino acids in SEQ ID NO:2,  $y$  is, for instance 0.70 for 70%, 0.80 for 80%, 0.85 for 85% etc., and  $\bullet$  is the symbol for the multiplication operator, and wherein any non-integer product of  $x_a$  and  $y$  is rounded down to the nearest integer prior to subtracting it from  $x_a$ .

"Individual(s)," when used herein with reference to an organism, means a multicellular eukaryote, including, but not limited to a metazoan, a mammal, an ovid, a bovid, a simian, a primate, and a human.

20

"Isolated" means altered "by the hand of man" from its natural state, *i.e.*, if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Moreover, a polynucleotide or polypeptide that is introduced into an organism by transformation, genetic manipulation or by any other recombinant method is "isolated" even if it is still present in said organism, which organism may be living or non-living.

25

(2) Polypeptide embodiments further include an isolated polypeptide comprising a polypeptide having at least a 50, 60, 70, 80, 85, 90, 95, 97 or 100% identity to a polypeptide reference sequence of SEQ ID NO:2, wherein said polypeptide sequence may be identical to the reference sequence of SEQ ID NO:2 or may include up to a certain integer number of amino acid alterations as compared to the reference sequence, wherein said alterations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence, and wherein said number of amino acid alterations is determined by multiplying the total number of amino acids in SEQ ID NO:2 by the integer defining the percent identity divided by 100 and then subtracting that product from said total number of amino acids in SEQ ID NO:2, or:

$$n_a \leq x_a - (x_a \bullet y),$$

wherein  $n_a$  is the number of amino acid alterations,  $x_a$  is the total number of amino acids in SEQ ID NO:2,  $y$  is 0.50 for 50%, 0.60 for 60%, 0.70 for 70%, 0.80 for 80%, 0.85 for 85%, 0.90 for 90%, 0.95 for 95%, 0.97 for 97% or 1.00 for 100%, and  $\bullet$  is the symbol for the multiplication operator, and wherein any non-integer product of  $x_a$  and  $y$  is rounded down to the nearest integer prior to subtracting it from  $x_a$ .

By way of example, a polypeptide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:2, that is it may be 100% identical, or it may include up to a certain integer number of amino acid alterations as compared to the reference sequence such that the percent identity is less than 100% identity. Such alterations are selected from the group consisting of at least one amino acid deletion,

wherein  $n_n$  is the number of nucleotide alterations,  $x_n$  is the total number of nucleotides in SEQ ID NO:1,  $y$  is 0.50 for 50%, 0.60 for 60%, 0.70 for 70%, 0.80 for 80%, 0.85 for 85%, 0.90 for 90%, 0.95 for 95%, 0.97 for 97% or 1.00 for 100%, and  $\bullet$  is the symbol for the multiplication operator, and wherein any non-integer product of  $x_n$  and  $y$  is rounded  
 5 down to the nearest integer prior to subtracting it from  $x_n$ . Alterations of a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2 may create nonsense, missense or frameshift mutations in this coding sequence and thereby alter the polypeptide encoded by the polynucleotide following such alterations.

10 By way of example, a polynucleotide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:1, that is it may be 100% identical, or it may include up to a certain integer number of nucleic acid alterations as compared to the reference sequence such that the percent identity is less than 100% identity. Such alterations are selected from the group consisting of at least one nucleic acid deletion,  
 15 substitution, including transition and transversion, or insertion, and wherein said alterations may occur at the 5' or 3' terminal positions of the reference polynucleotide sequence or anywhere between those terminal positions, interspersed either individually among the nucleic acids in the reference sequence or in one or more contiguous groups within the reference sequence. The number of nucleic acid alterations for a given percent  
 20 identity is determined by multiplying the total number of nucleic acids in SEQ ID NO:1 by the integer defining the percent identity divided by 100 and then subtracting that product from said total number of nucleic acids in SEQ ID NO:1, or:

$$n_n \leq x_n - (x_n \bullet y),$$

25

wherein  $n_n$  is the number of nucleic acid alterations,  $x_n$  is the total number of nucleic acids in SEQ ID NO:1,  $y$  is, for instance 0.70 for 70%, 0.80 for 80%, 0.85 for 85% etc.,  $\bullet$  is the symbol for the multiplication operator, and wherein any non-integer product of  $x_n$  and  $y$  is rounded down to the nearest integer prior to subtracting it from  $x_n$ .

Parameters for polynucleotide comparison include the following:

Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970)

Comparison matrix: matches = +10, mismatch = 0

Gap Penalty: 50

5 Gap Length Penalty: 3

Available as: The "gap" program from Genetics Computer Group, Madison WI. These are the default parameters for nucleic acid comparisons.

10 A preferred meaning for "identity" for polynucleotides and polypeptides, as the case may be, are provided in (1) and (2) below.

(1) Polynucleotide embodiments further include an isolated polynucleotide comprising a polynucleotide sequence having at least a 50, 60, 70, 80, 85, 90, 95, 97 or 100% identity to the reference sequence of SEQ ID NO:1, wherein said polynucleotide  
15 sequence may be identical to the reference sequence of SEQ ID NO:1 or may include up to a certain integer number of nucleotide alterations as compared to the reference sequence, wherein said alterations are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion, and wherein said alterations may occur at the 5' or 3' terminal positions of the reference  
20 nucleotide sequence or anywhere between those terminal positions, interspersed either individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence, and wherein said number of nucleotide alterations is determined by multiplying the total number of nucleotides in SEQ ID NO:1 by the integer defining the percent identity divided by 100 and then subtracting that  
25 product from said total number of nucleotides in SEQ ID NO:1, or:

$$n_n \leq x_n - (x_n \cdot y),$$

- methods, including but not limited to those described in (*Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heine, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., *SIAM J. Applied Math.*, 48: 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GAP program in the GCG program package (Devereux, J., et al., *Nucleic Acids Research* 12(1): 387 (1984)), BLASTP, BLASTN (Altschul, S.F. et al., *J. Mol. Biol.* 215: 403-410 (1990), and FASTA( Pearson and Lipman Proc. Natl. Acad. Sci. USA 85: 2444-2448 (1988). The BLAST family of programs is publicly available from NCBI and other sources (*BLAST Manual*, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894; Altschul, S., et al., *J. Mol. Biol.* 215: 403-410 (1990). The well known Smith Waterman algorithm may also be used to determine identity.
- Parameters for polypeptide sequence comparison include the following:
- Algorithm: Needleman and Wunsch, *J. Mol Biol.* 48: 443-453 (1970)
- Comparison matrix: BLOSSUM62 from Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992)
- Gap Penalty: 8
- Gap Length Penalty: 2
- A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison WI. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps).

nucleic acid base calling, codon usage analysis, nucleic acid base trimming, and sequencing chromatogram peak analysis.

5 A computer based method is provided for performing homology identification. This method comprises the steps of: providing a first polynucleotide sequence comprising the sequence of a polynucleotide of the invention in a computer readable medium; and comparing said first polynucleotide sequence to at least one second polynucleotide or polypeptide sequence to identify homology.

10 A computer based method is also provided for performing homology identification, said method comprising the steps of: providing a first polypeptide sequence comprising the sequence of a polypeptide of the invention in a computer readable medium; and comparing said first polypeptide sequence to at least one second polynucleotide or polypeptide sequence to identify homology.

15 All publications and references, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference in their entirety as if each individual publication or reference were specifically and individually indicated to be incorporated by reference herein as being fully set forth. Any patent  
20 application to which this application claims priority is also incorporated by reference herein in its entirety in the manner described above for publications and references.

## DEFINITIONS

25 "Identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as the case may be, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" can be readily calculated by known

A vaccine composition is conveniently in injectable form. Conventional adjuvants may be employed to enhance the immune response. A suitable unit dose for vaccination is 0.5-5 microgram/kg of antigen, and such dose is preferably administered 1-3 times and with an interval of 1-3 weeks. With the indicated dose range, no adverse toxicological effects will be observed with the compounds of the invention which would preclude their administration to suitable individuals.

Wide variations in the needed dosage, however, are to be expected in view of the variety of compounds available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art.

15

#### **Sequence Databases, Sequences in a Tangible Medium, and Algorithms**

Polynucleotide and polypeptide sequences form a valuable information resource with which to determine their 2- and 3-dimensional structures as well as to identify further sequences of similar homology. These approaches are most easily facilitated by storing the sequence in a computer readable medium and then using the stored data in a known macromolecular structure program or to search a sequence database using well known searching tools, such as the GCG program package.

Also provided by the invention are methods for the analysis of character sequences or strings, particularly genetic sequences or encoded protein sequences. Preferred methods of sequence analysis include, for example, methods of sequence homology analysis, such as identity and similarity analysis, DNA, RNA and protein structure analysis, sequence assembly, cladistic analysis, sequence motif analysis, open reading frame determination,

acceptable carrier or excipient. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The invention further relates to pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention.

- 5 Polypeptides, polynucleotides and other compounds of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

- The composition will be adapted to the route of administration, for instance by a systemic or an oral route. Preferred forms of systemic administration include injection, typically by  
10 intravenous injection. Other injection routes, such as subcutaneous, intramuscular, or intraperitoneal, can be used. Alternative means for systemic administration include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if a polypeptide or other compounds of the present invention can be formulated in an enteric or an encapsulated formulation, oral  
15 administration may also be possible. Administration of these compounds may also be topical and/or localized, in the form of salves, pastes, gels, solutions, powders and the like.

- For administration to mammals, and particularly humans, it is expected that the daily dosage level of the active agent will be from 0.01 mg/kg to 10 mg/kg, typically around 1  
20 mg/kg. The physician in any event will determine the actual dosage which will be most suitable for an individual and will vary with the age, weight and response of the particular individual. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

25

The dosage range required depends on the choice of peptide, the route of administration, the nature of the formulation, the nature of the subject's condition, and the judgment of the attending practitioner. Suitable dosages, however, are in the range of 0.1-100 µg/kg of subject.



The invention also relates to compositions comprising a polynucleotide and/or a polypeptide discussed herein or their agonists or antagonists. The polypeptides and polynucleotides of the invention may be employed in combination with a non-sterile or sterile carrier or carriers  
5 for use with cells, tissues or organisms, such as a pharmaceutical carrier suitable for administration to an individual. Such compositions comprise, for instance, a media additive or a therapeutically effective amount of a polypeptide and/or polynucleotide of the invention and a pharmaceutically acceptable carrier or excipient. Such carriers may include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol and combinations  
10 thereof. The formulation should suit the mode of administration. The invention further relates to diagnostic and pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention.

15 Polypeptides, polynucleotides and other compounds of the invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

The pharmaceutical compositions may be administered in any effective, convenient manner including, for instance, administration by topical, oral, anal, vaginal, intravenous,  
20 intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes among others.

In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

25

In a further aspect, the present invention provides for pharmaceutical compositions comprising a therapeutically effective amount of a polypeptide and/or polynucleotide, such as the soluble form of a polypeptide and/or polynucleotide of the present invention, agonist or antagonist peptide or small molecule compound, in combination with a pharmaceutically

may also be present at a level of 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain a stabiliser.

Non-toxic oil in water emulsions preferably contain a non-toxic oil, e.g. squalane or squalene, an emulsifier, e.g. Tween 80, in an aqueous carrier. The aqueous carrier may be, for example, phosphate buffered saline.

A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO 95/17210.

10

The present invention also provides a polyvalent vaccine composition comprising a vaccine formulation of the invention in combination with other antigens, in particular antigens useful for treating cancers, autoimmune diseases and related conditions. Such a polyvalent vaccine composition may include a TH-1 inducing adjuvant as hereinbefore described.

15

While the invention has been described with reference to certain BASB040 polypeptides and polynucleotides, it is to be understood that this covers fragments of the naturally occurring polypeptides and polynucleotides, and similar polypeptides and polynucleotides with additions, deletions or substitutions which do not substantially affect the immunogenic properties of the recombinant polypeptides or polynucleotides.

20

The antigen can also be delivered in the form of whole bacteria (dead or alive) or as subcellular fractions, these possibilities do include *N.meningitidis* itself.

25

#### Compositions, kits and administration

In a further aspect of the invention there are provided compositions comprising a BASB040 polynucleotide and/or a BASB040 polypeptide for administration to a cell or to a multicellular organism.

been shown to be successful TH1 stimulating adjuvants when formulated together with an antigen.

Further adjuvants which are preferential stimulators of TH1 cell response include  
5 immunomodulatory oligonucleotides, for example unmethylated CpG sequences as disclosed in WO 96/02555.

Combinations of different TH1 stimulating adjuvants, such as those mentioned hereinabove, are also contemplated as providing an adjuvant which is a preferential  
10 stimulator of TH1 cell response. For example, QS21 can be formulated together with 3D-MPL. The ratio of QS21 : 3D-MPL will typically be in the order of 1 : 10 to 10 : 1; preferably 1:5 to 5 : 1 and often substantially 1 : 1. The preferred range for optimal synergy is 2.5 : 1 to 1 : 1 3D-MPL: QS21.

15 Preferably a carrier is also present in the vaccine composition according to the invention. The carrier may be an oil in water emulsion, or an aluminium salt, such as aluminium phosphate or aluminium hydroxide.

A preferred oil-in-water emulsion comprises a metabolisable oil, such as squalene, alpha  
20 tocopherol and Tween 80. In a particularly preferred aspect the antigens in the vaccine composition according to the invention are combined with QS21 and 3D-MPL in such an emulsion. Additionally the oil in water emulsion may contain span 85 and/or lecithin and/or tricaprylin.

25 Typically for human administration QS21 and 3D-MPL will be present in a vaccine in the range of 1µg - 200µg, such as 10-100µg, preferably 10µg - 50µg per dose. Typically the oil in water will comprise from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% tween 80. Preferably the ratio of squalene: alpha tocopherol is equal to or less than 1 as this provides a more stable emulsion. Span 85

Thus, a TH1-type adjuvant is one which preferentially stimulates isolated T-cell populations to produce high levels of TH1-type cytokines when re-stimulated with antigen *in vitro*, and promotes development of both CD8+ cytotoxic T lymphocytes and antigen specific immunoglobulin responses associated with TH1-type isotype.

Adjuvants which are capable of preferential stimulation of the TH1 cell response are described in International Patent Application No. WO 94/00153 and WO 95/17209.

3 De-O-acylated monophosphoryl lipid A (3D-MPL) is one such adjuvant. This is known from GB 2220211 (Ribi). Chemically it is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem, Montana. A preferred form of 3 De-O-acylated monophosphoryl lipid A is disclosed in European Patent 0 689 454 B1 (SmithKline Beecham Biologicals SA).

Preferably, the particles of 3D-MPL are small enough to be sterile filtered through a 0.22micron membrane (European Patent number 0 689 454).

3D-MPL will be present in the range of 10µg - 100µg preferably 25-50µg per dose wherein the antigen will typically be present in a range 2-50µg per dose.

Another preferred adjuvant comprises QS21, an Hplc purified non-toxic fraction derived from the bark of Quillaja Saponaria Molina. Optionally this may be admixed with 3 De-O-acylated monophosphoryl lipid A (3D-MPL), optionally together with a carrier.

The method of production of QS21 is disclosed in US patent No. 5,057,540.

Non-reactogenic adjuvant formulations containing QS21 have been described previously (WO 96/33739). Such formulations comprising QS21 and cholesterol have

In mice TH1-type responses are often characterised by the generation of antibodies of the IgG2a subtype, whilst in the human these correspond to IgG1 type antibodies. TH2-type immune responses are characterised by the generation of a broad range of immunoglobulin isotypes including in mice IgG1, IgA, and IgM.

5

It can be considered that the driving force behind the development of these two types of immune responses are cytokines. High levels of TH1-type cytokines tend to favour the induction of cell mediated immune responses to the given antigen, whilst high levels of TH2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

10

The distinction of TH1 and TH2-type immune responses is not absolute. In reality an individual will support an immune response which is described as being predominantly TH1 or predominantly TH2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (*Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173*). Traditionally, TH1-type responses are associated with the production of the INF- $\gamma$  and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of TH1-type immune responses are not produced by T-cells, such as IL-12. In contrast, TH2-type responses are associated with the secretion of IL-4, IL-5, IL-6 and IL-13.

15

20

It is known that certain vaccine adjuvants are particularly suited to the stimulation of either TH1 or TH2 - type cytokine responses. Traditionally the best indicators of the TH1:TH2 balance of the immune response after a vaccination or infection includes direct measurement of the production of TH1 or TH2 cytokines by T lymphocytes *in vitro* after restimulation with antigen, and/or the measurement of the IgG1:IgG2a ratio of antigen specific antibody responses.

25

for the development of prophylactic agents or therapeutic treatments of bacterial infection, particularly *Neisseria meningitidis* infection, in mammals, particularly humans.

The invention also includes a vaccine formulation which comprises an immunogenic  
5 recombinant polypeptide and/or polynucleotide of the invention together with a suitable carrier, such as a pharmaceutically acceptable carrier. Since the polypeptides and polynucleotides may be broken down in the stomach, each is preferably administered parenterally, including, for example, administration that is subcutaneous, intramuscular, intravenous, or intradermal. Formulations suitable for parenteral administration include  
10 aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteristatic compounds and solutes which render the formulation isotonic with the bodily fluid, preferably the blood, of the individual; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed  
15 ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use.

The vaccine formulation of the invention may also include adjuvant systems for enhancing the immunogenicity of the formulation. Preferably the adjuvant system  
20 raises preferentially a TH1 type of response.

An immune response may be broadly distinguished into two extreme catagories, being a humoral or cell mediated immune responses (traditionally characterised by antibody and cellular effector mechanisms of protection respectively). These categories of response  
25 have been termed TH1-type responses (cell-mediated response), and TH2-type immune responses (humoral response).

Extreme TH1-type immune responses may be characterised by the generation of antigen specific, haplotype restricted cytotoxic T lymphocytes, and natural killer cell responses.

BASB040 gene. The upstream region starts immediately upstream of the BASB040 gene and continues usually to a position no more than about 1000 bp upstream of the gene from the ATG start codon. In the case of a gene located in a polycistronic sequence (operon) the upstream region can start immediately preceding the gene of interest, or preceding the first  
5 gene in the operon. Preferably, a modified upstream region according to this aspect of the invention contains a heterologous promotor at a position between 500 and 700 bp upstream of the ATG.

Thus, the invention provides a BASB040 polypeptide, in a modified bacterial bleb. The  
10 invention further provides modified host cells capable of producing the non-live membrane-based bleb vectors. The invention further provides nucleic acid vectors comprising the BASB040 gene having a modified upstream region containing a heterologous regulatory element.

15 Further provided by the invention are processes to prepare the host cells and bacterial blebs according to the invention.

Also provided by this invention are compositions, particularly vaccine compositions, and methods comprising the polypeptides and/or polynucleotides of the invention and  
20 immunostimulatory DNA sequences, such as those described in Sato, Y. *et al.* Science 273: 352 (1996).

Also, provided by this invention are methods using the described polynucleotide or particular fragments thereof, which have been shown to encode non-variable regions of  
25 bacterial cell surface proteins, in polynucleotide constructs used in such genetic immunization experiments in animal models of infection with *Neisseria meningitidis*. Such experiments will be particularly useful for identifying protein epitopes able to provoke a prophylactic or therapeutic immune response. It is believed that this approach will allow for the subsequent preparation of monoclonal antibodies of particular value,  
30 derived from the requisite organ of the animal successfully resisting or clearing infection.

sequences. In this case, the regulatory region targeted is isolated and modified so as to contain the regulatory elements from another gene, a combination of regulatory elements from different genes, a synthetic regulatory region, or any other regulatory region, or to delete selected parts of the wild-type regulatory sequences. These modified sequences can then be reintroduced into the bacterium via homologous recombination into the genome. A non-exhaustive list of preferred promoters that could be used for up-regulation of gene expression includes the promoters *porA*, *porB*, *lbpB*, *tbpB*, *p110*, *lst*, *hpuAB* from *N. meningitidis* or *N. gonorrhoeae*; *ompCD*, *copB*, *lbpB*, *ompE*, *UspA1*; *UspA2*; *TbpB* from *M. Catarrhalis*; *p1*, *p2*, *p4*, *p5*, *p6*, *lpD*, *tbpB*, *D15*, *Hia*, *Hmw1*, *Hmw2* from *H. influenzae*.

In one example, the expression of the gene can be modulated by exchanging its promoter with a stronger promoter (through isolating the upstream sequence of the gene, in vitro modification of this sequence, and reintroduction into the genome by homologous recombination). Upregulated expression can be obtained in both the bacterium as well as in the outer membrane vesicles shed (or made) from the bacterium.

In other examples, the described approaches can be used to generate recombinant bacterial strains with improved characteristics for vaccine applications. These can be, but are not limited to, attenuated strains, strains with increased expression of selected antigens, strains with knock-outs (or decreased expression) of genes interfering with the immune response, strains with modulated expression of immunodominant proteins, strains with modulated shedding of outer-membrane vesicles.

Thus, also provided by the invention is a modified upstream region of the BASB040 gene, particularly the part of the upstream region identified in Sequence ID No: 11, which modified upstream region contains a heterologous regulatory element which alters the expression level of the BASB040 protein located at the outer membrane. The upstream region according to this aspect of the invention includes the sequence upstream of the



- transcriptional and translational level. The sequence of these regions, either upstream or downstream of the open reading frame of the gene, can be obtained by DNA sequencing. This sequence information allows the determination of potential regulatory motifs such as the different promoter elements, terminator sequences, inducible sequence elements,
- 5 repressors, elements responsible for phase variation, the shine-dalgarno sequence, regions with potential secondary structure involved in regulation, as well as other types of regulatory motifs or sequences. A region directly upstream of the BASB040 gene is given in Sequence ID No: 11 This sequence is a further aspect of the invention.
- 10 This sequence information allows the modulation of the natural expression of the BASB040 gene. The upregulation of the gene expression may be accomplished by altering the promoter, the shine-dalgarno sequence, potential repressor or operator elements, or any other elements involved. Likewise, downregulation of expression can be achieved by similar types of modification. Alternatively, by changing phase variation
- 15 sequences, the expression of the gene can be put under phase variation control, or it may be uncoupled from this regulation. In another approach, the expression of the gene can be put under the control of one or more inducible elements allowing regulated expression. Examples of such regulation include, but are not limited to, induction by temperature shift, addition of inductor substrates like selected carbohydrates or their derivatives, trace
- 20 elements, vitamins, co-factors, metal ions, etc.

- Such modifications as described above can be introduced by several different means. The modification of sequences involved in gene expression can be carried out *in vivo* by random mutagenesis followed by selection for the desired phenotype. Another approach
- 25 consists in isolating the region of interest and modifying it by random mutagenesis, or site-directed replacement, insertion or deletion mutagenesis. The modified region can then be reintroduced into the bacterial genome by homologous recombination, and the effect on gene expression can be assessed. In another approach, the sequence knowledge of the region of interest can be used to replace or delete all or part of the natural regulatory

In a vaccine composition according to the invention, a BASB040 polypeptide and/or polynucleotide, or a fragment, or a mimotope, or a variant thereof may be present in a vector, such as the live recombinant vectors described above for example live bacterial  
5 vectors.

Also suitable are non-live vectors for the BASB040 polypeptide, for example bacterial outer-membrane vesicles or "blebs". OM blebs are derived from the outer membrane of the two-layer membrane of Gram-negative bacteria and have been documented in many  
10 Gram-negative bacteria (Zhou, L *et al.* 1998. *FEMS Microbiol. Lett.* 163:223-228) including *C. trachomatis* and *C. psittaci*. A non-exhaustive list of bacterial pathogens reported to produce blebs also includes: *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella melitensis*, *Brucella ovis*, *Escherichia coli*, *Haemophilus influenza*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*  
15 and *Yersinia enterocolitica*.

Blebs have the advantage of providing outer-membrane proteins in their native conformation and are thus particularly useful for vaccines. Blebs can also be improved for vaccine use by engineering the bacterium so as to modify the expression of one or  
20 more molecules at the outer membrane. Thus for example the expression of a desired immunogenic protein at the outer membrane, such as the BASB040 polypeptide, can be introduced or upregulated (e.g. by altering the promoter). Instead or in addition, the expression of outer-membrane molecules which are either not relevant (e.g. unprotective antigens or immunodominant but variable proteins) or detrimental (e.g. toxic molecules  
25 such as LPS, or potential inducers of an autoimmune response) can be downregulated. These approaches are discussed in more detail below.

The non-coding flanking regions of the BASB040 gene contain regulatory elements important in the expression of the gene. This regulation takes place both at the

antibody and/ or T cell immune response, including, for example, cytokine-producing T cells or cytotoxic T cells, to protect said individual, preferably a human, from disease, whether that disease is already established within the individual or not. One example of administering the gene is by accelerating it into the desired cells as a coating on particles  
5 or otherwise. Such nucleic acid vector may comprise DNA, RNA, a ribozyme, a modified nucleic acid, a DNA/RNA hybrid, a DNA-protein complex or an RNA-protein complex.

A further aspect of the invention relates to an immunological composition that when introduced into an individual, preferably a human, capable of having induced within it an  
10 immunological response, induces an immunological response in such individual to a BASB040 polynucleotide and/or polypeptide encoded therefrom, wherein the composition comprises a recombinant BASB040 polynucleotide and/or polypeptide encoded therefrom and/or comprises DNA and/or RNA which encodes and expresses an antigen of said  
15 BASB040 polynucleotide, polypeptide encoded therefrom, or other polypeptide of the invention. The immunological response may be used therapeutically or prophylactically and may take the form of antibody immunity and/or cellular immunity, such as cellular immunity arising from CTL or CD4+ T cells.

A BASB040 polypeptide or a fragment thereof may be fused with co-protein or chemical  
20 moiety which may or may not by itself produce antibodies, but which is capable of stabilizing the first protein and producing a fused or modified protein which will have antigenic and/or immunogenic properties, and preferably protective properties. Thus fused recombinant protein, preferably further comprises an antigenic co-protein, such as lipoprotein D from *Haemophilus influenzae*, Glutathione-S-transferase (GST) or beta-galactosidase, or any other relatively large co-protein which solubilizes the protein and  
25 facilitates production and purification thereof. Moreover, the co-protein may act as an adjuvant in the sense of providing a generalized stimulation of the immune system of the organism receiving the protein. The co-protein may be attached to either the amino- or carboxy-terminus of the first protein.

- distal from the conjugated terminus of the peptide, such that the free unconjugated end of the peptide remains associated with the surface of the carrier protein. Thereby presenting the peptide in a conformation which most closely resembles that of the peptide as found in the context of the whole native molecule. For example, the peptides
- 5 may be altered to have an N-terminal cysteine and a C-terminal hydrophobic amidated tail. Alternatively, the addition or substitution of a D-stereoisomer form of one or more of the amino acids may be performed to create a beneficial derivative, for example to enhance stability of the peptide.
- 10 Alternatively, peptide mimotopes may be identified using antibodies which are capable themselves of binding to the polypeptides of the present invention using techniques such as phage display technology (EP 0 552 267 B1). This technique, generates a large number of peptide sequences which mimic the structure of the native peptides and are, therefore, capable of binding to anti-native peptide antibodies, but may not necessarily themselves
- 15 share significant sequence homology to the native polypeptide.

### Vaccines

- Another aspect of the invention relates to a method for inducing an immunological response in an individual, particularly a mammal, preferably humans, which comprises
- 20 inoculating the individual with BASB040 polynucleotide and/or polypeptide, or a fragment or variant thereof, adequate to produce antibody and/ or T cell immune response to protect said individual from infection, particularly bacterial infection and most particularly *Neisseria meningitidis* infection. Also provided are methods whereby such immunological response slows bacterial replication. Yet another aspect of the invention
- 25 relates to a method of inducing immunological response in an individual which comprises delivering to such individual a nucleic acid vector, sequence or ribozyme to direct expression of BASB040 polynucleotide and/or polypeptide, or a fragment or a variant thereof, for expressing BASB040 polynucleotide and/or polypeptide, or a fragment or a variant thereof *in vivo* in order to induce an immunological response, such as, to produce

The invention also provides the use of the polypeptide, polynucleotide, agonist or antagonist of the invention to interfere with the initial physical interaction between a pathogen or pathogens and a eukaryotic, preferably mammalian, host responsible for sequelae of infection. In particular, the molecules of the invention may be used: in the  
5 prevention of adhesion of bacteria, in particular gram positive and/or gram negative bacteria, to eukaryotic, preferably mammalian, extracellular matrix proteins on in-dwelling devices or to extracellular matrix proteins in wounds; to block bacterial adhesion between eukaryotic, preferably mammalian, extracellular matrix proteins and bacterial  
BASB040 proteins that mediate tissue damage and/or; to block the normal progression of  
10 pathogenesis in infections initiated other than by the implantation of in-dwelling devices or by other surgical techniques.

In accordance with yet another aspect of the invention, there are provided BASB040 agonists and antagonists, preferably bacteristatic or bactericidal agonists and antagonists.  
15

The antagonists and agonists of the invention may be employed, for instance, to prevent, inhibit and/or treat diseases.

In a further aspect, the present invention relates to mimotopes of the polypeptide of the invention. A mimotope is a peptide sequence, sufficiently similar to the native peptide  
20 (sequentially or structurally), which is capable of being recognised by antibodies which recognise the native peptide; or is capable of raising antibodies which recognise the native peptide when coupled to a suitable carrier.

25 Peptide mimotopes may be designed for a particular purpose by addition, deletion or substitution of elected amino acids. Thus, the peptides may be modified for the purposes of ease of conjugation to a protein carrier. For example, it may be desirable for some chemical conjugation methods to include a terminal cysteine. In addition it may be desirable for peptides conjugated to a protein carrier to include a hydrophobic terminus

- Potential antagonists include a small molecule that binds to and occupies the binding site of the polypeptide thereby preventing binding to cellular binding molecules, such that normal biological activity is prevented. Examples of small molecules include but are not limited to small organic molecules, peptides or peptide-like molecules. Other potential antagonists
- 5 include antisense molecules (see Okano, *J. Neurochem.* 56: 560 (1991); *OLIGODEOXYNUCLEOTIDES AS ANTISENSE INHIBITORS OF GENE EXPRESSION*, CRC Press, Boca Raton, FL (1988), for a description of these molecules). Preferred potential antagonists include compounds related to and variants of BASB040.
- 10 In a further aspect, the present invention relates to genetically engineered soluble fusion proteins comprising a polypeptide of the present invention, or a fragment thereof, and various portions of the constant regions of heavy or light chains of immunoglobulins of various subclasses (IgG, IgM, IgA, IgE). Preferred as an immunoglobulin is the constant part of the heavy chain of human IgG, particularly IgG1, where fusion takes place at the
- 15 hinge region. In a particular embodiment, the Fc part can be removed simply by incorporation of a cleavage sequence which can be cleaved with blood clotting factor Xa. Furthermore, this invention relates to processes for the preparation of these fusion proteins by genetic engineering, and to the use thereof for drug screening, diagnosis and therapy. A further aspect of the invention also relates to polynucleotides encoding such
- 20 fusion proteins. Examples of fusion protein technology can be found in International Patent Application Nos. WO94/29458 and WO94/22914.

Each of the polynucleotide sequences provided herein may be used in the discovery and development of antibacterial compounds. The encoded protein, upon expression, can be

25 used as a target for the screening of antibacterial drugs. Additionally, the polynucleotide sequences encoding the amino terminal regions of the encoded protein or Shine-Delgarno or other translation facilitating sequences of the respective mRNA can be used to construct antisense sequences to control the expression of the coding sequence of interest.

reflected in decreased binding of the labeled ligand or decreased production of product from such substrate. Molecules that bind gratuitously, *i.e.*, without inducing the effects of BASB040 polypeptide are most likely to be good antagonists. Molecules that bind well and, as the case may be, increase the rate of product production from substrate, increase  
5 signal transduction, or increase chemical channel activity are agonists. Detection of the rate or level of, as the case may be, production of product from substrate, signal transduction, or chemical channel activity may be enhanced by using a reporter system. Reporter systems that may be useful in this regard include but are not limited to colorimetric, labeled substrate converted into product, a reporter gene that is responsive to changes in BASB040  
10 polynucleotide or polypeptide activity, and binding assays known in the art.

Another example of an assay for BASB040 agonists is a competitive assay that combines BASB040 and a potential agonist with BASB040-binding molecules, recombinant BASB040 binding molecules, natural substrates or ligands, or substrate or ligand mimetics,  
15 under appropriate conditions for a competitive inhibition assay. BASB040 can be labeled, such as by radioactivity or a colorimetric compound, such that the number of BASB040 molecules bound to a binding molecule or converted to product can be determined accurately to assess the effectiveness of the potential antagonist.

20 Potential antagonists include, among others, small organic molecules, peptides, polypeptides and antibodies that bind to a polynucleotide and/or polypeptide of the invention and thereby inhibit or extinguish its activity or expression. Potential antagonists also may be small organic molecules, a peptide, a polypeptide such as a closely related protein or antibody that binds the same sites on a binding molecule, such as a binding molecule, without inducing  
25 BASB040-induced activities, thereby preventing the action or expression of BASB040 polypeptides and/or polynucleotides by excluding BASB040 polypeptides and/or polynucleotides from binding.

may simply comprise the steps of mixing a candidate compound with a solution containing a polypeptide or polynucleotide of the present invention, to form a mixture, measuring BASB040 polypeptide and/or polynucleotide activity in the mixture, and comparing the BASB040 polypeptide and/or polynucleotide activity of the mixture to a standard. Fusion proteins, such as those made from Fc portion and BASB040 polypeptide, as hereinbefore described, can also be used for high-throughput screening assays to identify antagonists of the polypeptide of the present invention, as well as of phylogenetically and and/or functionally related polypeptides (see D. Bennett *et al.*, J Mol Recognition, 8:52-58 (1995); and K. Johanson *et al.*, J Biol Chem, 270(16):9459-9471 (1995)).

The polynucleotides, polypeptides and antibodies that bind to and/or interact with a polypeptide of the present invention may also be used to configure screening methods for detecting the effect of added compounds on the production of mRNA and/or polypeptide in cells. For example, an ELISA assay may be constructed for measuring secreted or cell associated levels of polypeptide using monoclonal and polyclonal antibodies by standard methods known in the art. This can be used to discover agents which may inhibit or enhance the production of polypeptide (also called antagonist or agonist, respectively) from suitably manipulated cells or tissues.

The invention also provides a method of screening compounds to identify those which enhance (agonist) or block (antagonist) the action of BASB040 polypeptides or polynucleotides, particularly those compounds that are bacteristatic and/or bactericidal. The method of screening may involve high-throughput techniques. For example, to screen for agonists or antagonists, a synthetic reaction mix, a cellular compartment, such as a membrane, cell envelope or cell wall, or a preparation of any thereof, comprising BASB040 polypeptide and a labeled substrate or ligand of such polypeptide is incubated in the absence or the presence of a candidate molecule that may be a BASB040 agonist or antagonist. The ability of the candidate molecule to agonize or antagonize the BASB040 polypeptide is



Preferably, the antibody or variant thereof is modified to make it less immunogenic in the individual. For example, if the individual is human the antibody may most preferably be "humanized," where the complementarity determining region or regions of the hybridoma-derived antibody has been transplanted into a human monoclonal antibody, for example as described in Jones *et al.* (1986), *Nature* 321, 522-525 or Tempest *et al.*, (1991) *Biotechnology* 9, 266-273.

#### Antagonists and Agonists - Assays and Molecules

10 Polypeptides and polynucleotides of the invention may also be used to assess the binding of small molecule substrates and ligands in, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. These substrates and ligands may be natural substrates and ligands or may be structural or functional mimetics. See, *e.g.*, Coligan *et al.*, *Current Protocols in Immunology* 1(2): Chapter 5 (1991).

15 The screening methods may simply measure the binding of a candidate compound to the polypeptide or polynucleotide, or to cells or membranes bearing the polypeptide or polynucleotide, or a fusion protein of the polypeptide by means of a label directly or indirectly associated with the candidate compound. Alternatively, the screening method may involve competition with a labeled competitor. Further, these screening methods may test whether the candidate compound results in a signal generated by activation or inhibition of the polypeptide or polynucleotide, using detection systems appropriate to the cells comprising the polypeptide or polynucleotide. Inhibitors of activation are generally assayed in the presence of a known agonist and the effect on activation by the agonist by the presence of the candidate compound is observed. Constitutively active polypeptide and/or constitutively expressed polypeptides and polynucleotides may be employed in screening methods for inverse agonists or inhibitors, in the absence of an agonist or inhibitor, by testing whether the candidate compound results in inhibition of activation of the polypeptide or polynucleotide, as the case may be. Further, the screening methods

preparation of monoclonal antibodies, any technique known in the art that provides antibodies produced by continuous cell line cultures can be used. Examples include various techniques, such as those in Kohler, G. and Milstein, C., *Nature* 256: 495-497 (1975); Kozbor *et al.*, *Immunology Today* 4: 72 (1983); Cole *et al.*, pg. 77-96 in *MONOCLONAL*  
5 *ANTIBODIES AND CANCER THERAPY*, Alan R. Liss, Inc. (1985).

Techniques for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce single chain antibodies to polypeptides or polynucleotides of this invention. Also, transgenic mice, or other organisms or animals, such as other mammals,  
10 may be used to express humanized antibodies immunospecific to the polypeptides or polynucleotides of the invention.

Alternatively, phage display technology may be utilized to select antibody genes with binding activities towards a polypeptide of the invention either from repertoires of PCR  
15 amplified v-genes of lymphocytes from humans screened for possessing anti-BASB040 or from naive libraries (McCafferty, *et al.*, (1990), *Nature* 348, 552-554; Marks, *et al.*, (1992) *Biotechnology* 10, 779-783). The affinity of these antibodies can also be improved by, for example, chain shuffling (Clackson *et al.*, (1991) *Nature* 352: 628).

20 The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptides or polynucleotides of the invention to purify the polypeptides or polynucleotides by, for example, affinity chromatography.

Thus, among others, antibodies against BASB040-polypeptide or BASB040-polynucleotide  
25 may be employed to treat infections, particularly bacterial infections.

Polypeptide variants include antigenically, epitopically or immunologically equivalent variants form a particular aspect of this invention.

radioimmunoassays, competitive-binding assays, Western Blot analysis, antibody sandwich assays, antibody detection and ELISA assays.

The polynucleotides of the invention may be used as components of polynucleotide  
5 arrays, preferably high density arrays or grids. These high density arrays are particularly useful for diagnostic and prognostic purposes. For example, a set of spots each comprising a different gene, and further comprising a polynucleotide or polynucleotides of the invention, may be used for probing, such as using hybridization or nucleic acid amplification, using a probe obtained or derived from a bodily sample, to  
10 determine the presence of a particular polynucleotide sequence or related sequence in an individual. Such a presence may indicate the presence of a pathogen, particularly *Neisseria meningitidis*, and may be useful in diagnosing and/or prognosing disease or a course of disease. A grid comprising a number of variants of the polynucleotide sequence of SEQ ID NO:1, 3, 5 are preferred. Also preferred is a grid comprising a  
15 number of variants of a polynucleotide sequence encoding the polypeptide sequence of SEQ ID NO:2, 4, 6.

### Antibodies

The polypeptides and polynucleotides of the invention or variants thereof, or cells  
20 expressing the same can be used as immunogens to produce antibodies immunospecific for such polypeptides or polynucleotides respectively.

In certain preferred embodiments of the invention there are provided antibodies against  
25 BASB040 polypeptides or polynucleotides.

Antibodies generated against the polypeptides or polynucleotides of the invention can be obtained by administering the polypeptides and/or polynucleotides of the invention, or epitope-bearing fragments of either or both, analogues of either or both, or cells expressing either or both, to an animal, preferably a nonhuman, using routine protocols. For

example. For example, RT-PCR can be used to detect mutations in the RNA. It is particularly preferred to use RT-PCR in conjunction with automated detection systems, such as, for example, GeneScan. RNA, cDNA or genomic DNA may also be used for the same purpose, PCR. As an example, PCR primers complementary to a polynucleotide encoding  
5 BASB040 polypeptide can be used to identify and analyze mutations.

The invention further provides primers with 1, 2, 3 or 4 nucleotides removed from the 5' and/or the 3' end. These primers may be used for, among other things, amplifying  
10 BASB040 DNA and/or RNA isolated from a sample derived from an individual, such as a bodily material. The primers may be used to amplify a polynucleotide isolated from an infected individual, such that the polynucleotide may then be subject to various techniques for elucidation of the polynucleotide sequence. In this way, mutations in the polynucleotide sequence may be detected and used to diagnose and/or prognose the infection or its stage or course, or to serotype and/or classify the infectious agent.

15 The invention further provides a process for diagnosing disease, preferably bacterial infections, more preferably infections caused by *Neisseria meningitidis*, comprising determining from a sample derived from an individual, such as a bodily material, an increased level of expression of polynucleotide having a sequence of SEQ ID NO:1, 3, 5.  
20 Increased or decreased expression of a BASB040 polynucleotide can be measured using any one of the methods well known in the art for the quantitation of polynucleotides, such as, for example, amplification, PCR, RT-PCR, RNase protection, Northern blotting, spectrometry and other hybridization methods.

25 In addition, a diagnostic assay in accordance with the invention for detecting over-expression of BASB040 polypeptide compared to normal control tissue samples may be used to detect the presence of an infection, for example. Assay techniques that can be used to determine levels of a BASB040 polypeptide, in a sample derived from a host, such as a bodily material, are well-known to those of skill in the art. Such assay methods include

address a variety of questions in molecular genetics including gene expression, genetic linkage, and genetic variability (see, for example, Chee *et al.*, *Science*, 274: 610 (1996)).

Thus in another aspect, the present invention relates to a diagnostic kit which comprises:

- 5 (a) a polynucleotide of the present invention, preferably the nucleotide sequence of SEQ ID NO:1, 3, 5, or a fragment thereof;
- (b) a nucleotide sequence complementary to that of (a);
- (c) a polypeptide of the present invention, preferably the polypeptide of SEQ ID NO:2, 4, 6 or a fragment thereof; or
- 10 (d) an antibody to a polypeptide of the present invention, preferably to the polypeptide of SEQ ID NO:2, 4, 6.

It will be appreciated that in any such kit, (a), (b), (c) or (d) may comprise a substantial component. Such a kit will be of use in diagnosing a disease or susceptibility to a disease,  
15 among others.

This invention also relates to the use of polynucleotides of the present invention as diagnostic reagents. Detection of a mutated form of a polynucleotide of the invention, preferable, SEQ ID NO:1, 3, 5, which is associated with a disease or pathogenicity will  
20 provide a diagnostic tool that can add to, or define, a diagnosis of a disease, a prognosis of a course of disease, a determination of a stage of disease, or a susceptibility to a disease, which results from under-expression, over-expression or altered expression of the polynucleotide. Organisms, particularly infectious organisms, carrying mutations in such polynucleotide may be detected at the polynucleotide level by a variety of techniques, such  
25 as those described elsewhere herein.

Cells from an organism carrying mutations or polymorphisms (allelic variations) in a polynucleotide and/or polypeptide of the invention may also be detected at the polynucleotide or polypeptide level by a variety of techniques, to allow for serotyping, for

- Polypeptides and polynucleotides for prognosis, diagnosis or other analysis may be obtained from a putatively infected and/or infected individual's bodily materials. Polynucleotides from any of these sources, particularly DNA or RNA, may be used directly for detection or
- 5 may be amplified enzymatically by using PCR or any other amplification technique prior to analysis. RNA, particularly mRNA, cDNA and genomic DNA may also be used in the same ways. Using amplification, characterization of the species and strain of infectious or resident organism present in an individual, may be made by an analysis of the genotype of a selected polynucleotide of the organism. Deletions and insertions can be detected by a
- 10 change in size of the amplified product in comparison to a genotype of a reference sequence selected from a related organism, preferably a different species of the same genus or a different strain of the same species. Point mutations can be identified by hybridizing amplified DNA to labeled BASB040 polynucleotide sequences. Perfectly or significantly matched sequences can be distinguished from imperfectly or more significantly mismatched
- 15 duplexes by DNase or RNase digestion, for DNA or RNA respectively, or by detecting differences in melting temperatures or renaturation kinetics. Polynucleotide sequence differences may also be detected by alterations in the electrophoretic mobility of polynucleotide fragments in gels as compared to a reference sequence. This may be carried out with or without denaturing agents. Polynucleotide differences may also be detected by
- 20 direct DNA or RNA sequencing. See, for example, Myers *et al.*, *Science*, 230: 1242 (1985). Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase, V1 and S1 protection assay or a chemical cleavage method. See, for example, Cotton *et al.*, *Proc. Natl. Acad. Sci., USA*, 85: 4397-4401 (1985).
- 25 In another embodiment, an array of oligonucleotides probes comprising BASB040 nucleotide sequence or fragments thereof can be constructed to conduct efficient screening of, for example, genetic mutations, serotype, taxonomic classification or identification. Array technology methods are well known and have general applicability and can be used to

- Polypeptides of the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, ion metal affinity chromatography (IMAC) is employed for purification. Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during intracellular synthesis, isolation and or purification.
- The expression system may also be a recombinant live microorganism, such as a virus or bacterium. The gene of interest can be inserted into the genome of a live recombinant virus or bacterium. Inoculation and *in vivo* infection with this live vector will lead to *in vivo* expression of the antigen and induction of immune responses. Viruses and bacteria used for this purpose are for instance: poxviruses (e.g; vaccinia, fowlpox, canarypox), alphaviruses (Sindbis virus, Semliki Forest Virus, Venezuelan Equine Encephalitis Virus), adenoviruses, adeno-associated virus, picornaviruses (poliovirus, rhinovirus), herpesviruses (varicella zoster virus, etc), Listeria, Salmonella, Shigella, Neisseria, BCG. These viruses and bacteria can be virulent, or attenuated in various ways in order to obtain live vaccines. Such live vaccines also form part of the invention.

20

#### Diagnostic, Prognostic, Serotyping and Mutation Assays

- This invention is also related to the use of BASB040 polynucleotides and polypeptides of the invention for use as diagnostic reagents. Detection of BASB040 polynucleotides and/or polypeptides in a eukaryote, particularly a mammal, and especially a human, will provide a diagnostic method for diagnosis of disease, staging of disease or response of an infectious organism to drugs. Eukaryotes, particularly mammals, and especially humans, particularly those infected or suspected to be infected with an organism comprising the BASB040 gene or protein, may be detected at the nucleic acid or amino acid level by a variety of well known techniques as well as by methods provided herein.

Representative examples of appropriate hosts include bacterial cells, such as cells of streptococci, staphylococci, enterococci, *E. coli*, streptomyces, cyanobacteria, *Bacillus subtilis*, *Moraxella catarrhalis*, *Haemophilus influenzae* and *Neisseria meningitidis*; fungal cells, such as cells of a yeast, *Kluyveromyces*, *Saccharomyces*, a basidiomycete, *Candida albicans* and *Aspergillus*; insect cells such as cells of *Drosophila* S2 and *Spodoptera* Sf9; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293, CV-1 and Bowes melanoma cells; and plant cells, such as cells of a gymnosperm or angiosperm.

A great variety of expression systems can be used to produce the polypeptides of the invention. Such vectors include, among others, chromosomal-, episomal- and virus-derived vectors, for example, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses, picornaviruses, retroviruses, and alphaviruses and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression system constructs may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides and/or to express a polypeptide in a host may be used for expression in this regard. The appropriate DNA sequence may be inserted into the expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, *MOLECULAR CLONING, A LABORATORY MANUAL*, (*supra*).

In recombinant expression systems in eukaryotes, for secretion of a translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signals.



*Nature* (1992) 356:152, Eisenbraun *et al.*, *DNA Cell Biol* (1993) 12: 791) and *in vivo* infection using cloned retroviral vectors (Seeger *et al.*, *PNAS USA* (1984) 81: 5849).

5    **Vectors, Host Cells, Expression Systems**

The invention also relates to vectors that comprise a polynucleotide or polynucleotides of the invention, host cells that are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived  
10    from the DNA constructs of the invention.

Recombinant polypeptides of the present invention may be prepared by processes well known in those skilled in the art from genetically engineered host cells comprising expression systems. Accordingly, in a further aspect, the present invention relates to  
15    expression systems that comprise a polynucleotide or polynucleotides of the present invention, to host cells which are genetically engineered with such expression systems, and to the production of polypeptides of the invention by recombinant techniques.

For recombinant production of the polypeptides of the invention, host cells can be  
20    genetically engineered to incorporate expression systems or portions thereof or polynucleotides of the invention. Introduction of a polynucleotide into the host cell can be effected by methods described in many standard laboratory manuals, such as Davis, *et al.*, *BASIC METHODS IN MOLECULAR BIOLOGY*, (1986) and Sambrook, *et al.*, *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor  
25    Laboratory Press, Cold Spring Harbor, N.Y. (1989), such as, calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction and infection.

such inactive precursors generally are activated. Some or all of the prosequences may be removed before activation. Generally, such precursors are called proproteins.

5 In addition to the standard A, G, C, T/U representations for nucleotides, the term "N" may also be used in describing certain polynucleotides of the invention. "N" means that any of the four DNA or RNA nucleotides may appear at such a designated position in the DNA or RNA sequence, except it is preferred that N is not a nucleic acid that when taken in combination with adjacent nucleotide positions, when read in the correct reading frame, would have the effect of generating a premature termination codon in such reading frame.

10

In sum, a polynucleotide of the invention may encode a mature protein, a mature protein plus a leader sequence (which may be referred to as a preprotein), a precursor of a mature protein having one or more prosequences that are not the leader sequences of a preprotein, or a preproprotein, which is a precursor to a proprotein, having a leader sequence and one or  
15 more prosequences, which generally are removed during processing steps that produce active and mature forms of the polypeptide.

In accordance with an aspect of the invention, there is provided the use of a polynucleotide of the invention for therapeutic or prophylactic purposes, in particular  
20 genetic immunization.

The use of a polynucleotide of the invention in genetic immunization will preferably employ a suitable delivery method such as direct injection of plasmid DNA into muscles (Wolff *et al.*, *Hum Mol Genet* (1992) 1: 363, Manthorpe *et al.*, *Hum. Gene Ther.* (1983) 4:  
25 419), delivery of DNA complexed with specific protein carriers (Wu *et al.*, *J Biol Chem.* (1989) 264: 16985), coprecipitation of DNA with calcium phosphate (Benvenisty & Reshef, *PNAS USA*, (1986) 83: 9551), encapsulation of DNA in various forms of liposomes (Kaneda *et al.*, *Science* (1989) 243: 375), particle bombardment (Tang *et al.*,

to the existing DNA to give a complete sequence, or carrying out a separate full-length PCR using the new sequence information for the design of the 5' primer.

5 The polynucleotides and polypeptides of the invention may be employed, for example, as research reagents and materials for discovery of treatments of and diagnostics for diseases, particularly human diseases, as further discussed herein relating to polynucleotide assays.

10 The polynucleotides of the invention that are oligonucleotides derived from a sequence of SEQ ID NOS:1 – 6 may be used in the processes herein as described, but preferably for PCR, to determine whether or not the polynucleotides identified herein in whole or in part are transcribed in bacteria in infected tissue. It is recognized that such sequences will also have utility in diagnosis of the stage of infection and type of infection the pathogen has attained.

15 The invention also provides polynucleotides that encode a polypeptide that is the mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids interior to the mature polypeptide (when the mature form has more than one polypeptide chain, for instance). Such sequences may play a role in processing of a protein from precursor to a mature form, may allow protein transport, may lengthen or shorten protein half-life or may  
20 facilitate manipulation of a protein for assay or production, among other things. As generally is the case *in vivo*, the additional amino acids may be processed away from the mature protein by cellular enzymes.

25 For each and every polynucleotide of the invention there is provided a polynucleotide complementary to it. It is preferred that these complementary polynucleotides are fully complementary to each polynucleotide with which they are complementary.

A precursor protein, having a mature form of the polypeptide fused to one or more prosequences may be an inactive form of the polypeptide. When prosequences are removed

RNA, cDNA and genomic DNA to isolate full-length cDNAs and genomic clones encoding BASB040 and to isolate cDNA and genomic clones of other genes that have a high identity, particularly high sequence identity, to the BASB040 gene. Such probes generally will comprise at least 15 nucleotide residues or base pairs. Preferably, such probes will have at least 30 nucleotide residues or base pairs and may have at least 50 nucleotide residues or base pairs. Particularly preferred probes will have at least 20 nucleotide residues or base pairs and will have less than 30 nucleotide residues or base pairs.

A coding region of a BASB040 gene may be isolated by screening using a DNA sequence provided in SEQ ID NO:1, 3, 5 to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the invention is then used to screen a library of cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

There are several methods available and well known to those skilled in the art to obtain full-length DNAs, or extend short DNAs, for example those based on the method of Rapid Amplification of cDNA ends (RACE) (see, for example, Frohman, *et al.*, *PNAS USA* 85: 8998-9002, 1988). Recent modifications of the technique, exemplified by the Marathon™ technology (Clontech Laboratories Inc.) for example, have significantly simplified the search for longer cDNAs. In the Marathon™ technology, cDNAs have been prepared from mRNA extracted from a chosen tissue and an 'adaptor' sequence ligated onto each end. Nucleic acid amplification (PCR) is then carried out to amplify the "missing" 5' end of the DNA using a combination of gene specific and adaptor specific oligonucleotide primers. The PCR reaction is then repeated using "nested" primers, that is, primers designed to anneal within the amplified product (typically an adaptor specific primer that anneals further 3' in the adaptor sequence and a gene specific primer that anneals further 5' in the selected gene sequence). The products of this reaction can then be analyzed by DNA sequencing and a full-length DNA constructed either by joining the product directly

In accordance with certain preferred embodiments of this invention there are provided polynucleotides that hybridize, particularly under stringent conditions, to BASB040 polynucleotide sequences, such as those polynucleotides in SEQ ID NO:1, 3, 5.

- 5 The invention further relates to polynucleotides that hybridize to the polynucleotide sequences provided herein. In this regard, the invention especially relates to polynucleotides that hybridize under stringent conditions to the polynucleotides described herein. As herein used, the terms "stringent conditions" and "stringent hybridization conditions" mean hybridization occurring only if there is at least 95% and preferably at least 97% identity
- 10 between the sequences. A specific example of stringent hybridization conditions is overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 micrograms/ml of denatured, sheared salmon sperm DNA, followed by washing the hybridization support in 0.1x SSC at about 65°C.
- 15 Hybridization and wash conditions are well known and exemplified in Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), particularly Chapter 11 therein. Solution hybridization may also be used with the polynucleotide sequences provided by the invention.
- 20 The invention also provides a polynucleotide consisting of or comprising a polynucleotide sequence obtained by screening an appropriate library containing the complete gene for a polynucleotide sequence set forth in SEQ ID NO:1, 3, 5 under stringent hybridization conditions with a probe having the sequence of said polynucleotide sequence set forth in SEQ ID NO:1, 3, 5 or a fragment thereof; and isolating said polynucleotide sequence.
- 25 Fragments useful for obtaining such a polynucleotide include, for example, probes and primers fully described elsewhere herein.

As discussed elsewhere herein regarding polynucleotide assays of the invention, for instance, the polynucleotides of the invention, may be used as a hybridization probe for

integrated transposon sequence, or due to RNA editing or genomic DNA reorganization) together with additional regions, that also may contain coding and/or non-coding sequences.

The invention further relates to variants of the polynucleotides described herein that encode  
5 variants of a polypeptide having a deduced amino acid sequence of SEQ ID NO:2, 4, 6.  
Fragments of polynucleotides of the invention may be used, for example, to synthesize full-length polynucleotides of the invention.

Further particularly preferred embodiments are polynucleotides encoding BASB040  
10 variants, that have the amino acid sequence of BASB040 polypeptide of SEQ ID NO:2, 4, 6  
in which several, a few, 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues are substituted,  
modified, deleted and/or added, in any combination. Especially preferred among these are  
silent substitutions, additions and deletions, that do not alter the properties and activities of  
BASB040 polypeptide.

15  
Further preferred embodiments of the invention are polynucleotides that are at least 85%  
identical over their entire length to a polynucleotide encoding BASB040 polypeptide having  
an amino acid sequence set out in SEQ ID NO:2, 4, 6, and polynucleotides that are  
complementary to such polynucleotides. In this regard, polynucleotides at least 90%  
20 identical over their entire length to the same are particularly preferred, and among these  
particularly preferred polynucleotides, those with at least 95% are especially preferred.  
Furthermore, those with at least 97% are highly preferred among those with at least 95%,  
and among these those with at least 98% and at least 99% are particularly highly preferred,  
with at least 99% being the more preferred.

25  
Preferred embodiments are polynucleotides encoding polypeptides that retain substantially  
the same biological function or activity as the mature polypeptide encoded by a DNA of  
SEQ ID NO:1, 3, 5.

non-coding sequence, including for example, but not limited to at least one non-coding 5' and 3' sequence, such as the transcribed but non-translated sequences, termination signals (such as rho-dependent and rho-independent termination signals), ribosome binding sites, Kozak sequences, sequences that stabilize mRNA, introns, and polyadenylation signals.

5 The polynucleotide sequence may also comprise additional coding sequence encoding additional amino acids. For example, a marker sequence that facilitates purification of the fused polypeptide can be encoded. In certain embodiments of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz *et al.*, *Proc. Natl. Acad. Sci., USA* 86: 821-824 (1989), or an HA peptide tag (Wilson *et al.*, *Cell* 37: 767 (1984), both of which may be useful in purifying  
10 polypeptide sequence fused to them. Polynucleotides of the invention also include, but are not limited to, polynucleotides comprising a structural gene and its naturally associated sequences that control gene expression.

15 The nucleotide sequence encoding BASB040 polypeptide of SEQ ID NO:2, 4, 6 may be identical to the polypeptide encoding sequence contained in nucleotides 1 to 1827 of SEQ ID NO:1, or the polypeptide encoding sequence contained in nucleotides 1 to 1827 of SEQ ID NO:3, or the polypeptide encoding sequence contained in nucleotides 1 to 1761 of SEQ ID NO:5, respectively. Alternatively it may be a sequence, which as a result of the  
20 redundancy (degeneracy) of the genetic code, also encodes the polypeptide of SEQ ID NO:2, 4, 6.

The term "polynucleotide encoding a polypeptide" as used herein encompasses polynucleotides that include a sequence encoding a polypeptide of the invention,  
25 particularly a bacterial polypeptide and more particularly a polypeptide of the *Neisseria meningitidis* BASB040 having an amino acid sequence set out in SEQ ID NO:2, 4, 6. The term also encompasses polynucleotides that include a single continuous region or discontinuous regions encoding the polypeptide (for example, polynucleotides interrupted by integrated phage, an integrated insertion sequence, an integrated vector sequence, an

The polynucleotide of SEQ ID NO:5, between the first codon at nucleotide number 1 and the stop codon which begins at nucleotide number 1762 of SEQ ID NO:5, encodes the polypeptide of SEQ ID NO:6.

- 5 In a further aspect, the present invention provides for an isolated polynucleotide comprising or consisting of:
- (a) a polynucleotide sequence which has at least 85% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, even more preferably at least 97-99% or exact identity to SEQ ID NO:1,3,5 over the entire length of SEQ ID
  - 10 NO:1,3,5 respectively; or
  - (b) a polynucleotide sequence encoding a polypeptide which has at least 85% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, even more preferably at least 97-99% or 100% exact, to the amino acid sequence of SEQ ID NO:2, 4, 6 over the entire length of SEQ ID NO:2, 4, 6 respectively.
- 15
- A polynucleotide encoding a polypeptide of the present invention, including homologs and orthologs from species other than *Neisseria meningitidis*, may be obtained by a process which comprises the steps of screening an appropriate library under stringent hybridization conditions (for example, using a temperature in the range of 45 – 65°C and an SDS
- 20 concentration from 0.1 – 1%) with a labeled or detectable probe consisting of or comprising the sequence of SEQ ID NO: 1, 3, 5 or a fragment thereof; and isolating a full-length gene and/or genomic clones containing said polynucleotide sequence.

- The invention provides a polynucleotide sequence identical over its entire length to a coding
- 25 sequence (open reading frame) in SEQ ID NO: 1, 3, 5. Also provided by the invention is a coding sequence for a mature polypeptide or a fragment thereof, by itself as well as a coding sequence for a mature polypeptide or a fragment in reading frame with another coding sequence, such as a sequence encoding a leader or secretory sequence, a pre-, or pro- or prepro-protein sequence. The polynucleotide of the invention may also contain at least one



SEQ ID NO:1,3,5, typically a library of clones of chromosomal DNA of *Neisseria meningitidis* in *E.coli* or some other suitable host is probed with a radiolabeled oligonucleotide, preferably a 17-mer or longer, derived from a partial sequence. Clones carrying DNA identical to that of the probe can then be distinguished using stringent hybridization conditions. By sequencing the individual clones thus identified by hybridization with sequencing primers designed from the original polypeptide or polynucleotide sequence it is then possible to extend the polynucleotide sequence in both directions to determine a full length gene sequence. Conveniently, such sequencing is performed, for example, using denatured double stranded DNA prepared from a plasmid clone. Suitable techniques are described by Maniatis, T., Fritsch, E.F. and Sambrook et al., *MOLECULAR CLONING, A LABORATORY MANUAL*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989). (see in particular Screening By Hybridization 1.90 and Sequencing Denatured Double-Stranded DNA Templates 13.70). Direct genomic DNA sequencing may also be performed to obtain a full length gene sequence. Illustrative of the invention, each polynucleotide set out in SEQ ID NO:1,3,5 was discovered in a DNA library derived from *Neisseria meningitidis*.

Moreover, each DNA sequence set out in SEQ ID NO:1,3,5 contains an open reading frame encoding a protein having about the number of amino acid residues set forth in SEQ ID NO:2, 4, 6 with a deduced molecular weight that can be calculated using amino acid residue molecular weight values well known to those skilled in the art.

The polynucleotide of SEQ ID NO:1, between the start codon at nucleotide number 1 and the stop codon which begins at nucleotide number 1828 of SEQ ID NO:1, encodes the polypeptide of SEQ ID NO:2.

The polynucleotide of SEQ ID NO:3, between the start codon at nucleotide number 1 and the stop codon which begins at nucleotide number 1828 of SEQ ID NO:3, encodes the polypeptide of SEQ ID NO:4.

In a particularly preferred embodiment of the invention the polynucleotide comprises a region encoding BASB040 polypeptides comprising a sequence set out in SEQ ID NO:1,3,5 which includes a full length gene, or a variant thereof.

- 5 The BASB040 polynucleotides provided in SEQ ID NO:1,3 and 5 are the BASB040 polynucleotides from *Neisseria meningitidis* strains ATCC13090 and H44/76.

As a further aspect of the invention there are provided isolated nucleic acid molecules encoding and/or expressing BASB040 polypeptides and polynucleotides, particularly  
10 *Neisseria meningitidis* BASB040 polypeptides and polynucleotides, including, for example, unprocessed RNAs, ribozyme RNAs, mRNAs, cDNAs, genomic DNAs, B- and Z-DNAs. Further embodiments of the invention include biologically, diagnostically, prophylactically, clinically or therapeutically useful polynucleotides and polypeptides, and variants thereof, and compositions comprising the same.

15

Another aspect of the invention relates to isolated polynucleotides, including at least one full length gene, that encodes a BASB040 polypeptide having a deduced amino acid sequence of SEQ ID NO:2,4,6 and polynucleotides closely related thereto and variants thereof.

- 20 In another particularly preferred embodiment of the invention there is a BASB040 polypeptide from *Neisseria meningitidis* comprising or consisting of an amino acid sequence of SEQ ID NO:2,4,6 or a variant thereof.

Using the information provided herein, such as a polynucleotide sequence set out in SEQ ID  
25 NO:1, 3, 5 a polynucleotide of the invention encoding BASB040 polypeptide may be obtained using standard cloning and screening methods, such as those for cloning and sequencing chromosomal DNA fragments from bacteria using *Neisseria meningitidis* cells as starting material, followed by obtaining a full length clone. For example, to obtain a polynucleotide sequence of the invention, such as a polynucleotide sequence given in

expression of fusion proteins. Purification of hybrid proteins containing the C-LytA fragment at its amino terminus has been described {Biotechnology: 10, (1992) page 795-798}. It is possible to use the repeat portion of the LytA molecule found in the C terminal end starting at residue 178, for example residues 188 - 305.

5

The present invention also includes variants of the aforementioned polypeptides, that is polypeptides that vary from the referents by conservative amino acid substitutions, whereby a residue is substituted by another with like characteristics. Typical such substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr.

10

Polypeptides of the present invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

15

It is most preferred that a polypeptide of the invention is derived from *Neisseria meningitidis*, however, it may preferably be obtained from other organisms of the same taxonomic genus. A polypeptide of the invention may also be obtained, for example, from organisms of the same taxonomic family or order.

20

#### Polynucleotides

It is an object of the invention to provide polynucleotides that encode BASB040 polypeptides, particularly polynucleotides that encode the polypeptide herein designated BASB040.

25

portions of the constant regions of heavy or light chains of immunoglobulins of various subclasses (IgG, IgM, IgA, IgE). Preferred as an immunoglobulin is the constant part of the heavy chain of human IgG, particularly IgG1, where fusion takes place at the hinge region. In a particular embodiment, the Fc part can be removed simply by incorporation  
5 of a cleavage sequence which can be cleaved with blood clotting factor Xa.

Furthermore, this invention relates to processes for the preparation of these fusion proteins by genetic engineering, and to the use thereof for drug screening, diagnosis and therapy. A further aspect of the invention also relates to polynucleotides encoding such  
10 fusion proteins. Examples of fusion protein technology can be found in International Patent Application Nos. WO94/29458 and WO94/22914.

The proteins may be chemically conjugated, or expressed as recombinant fusion proteins allowing increased levels to be produced in an expression system as compared  
15 to non-fused protein. The fusion partner may assist in providing T helper epitopes (immunological fusion partner), preferably T helper epitopes recognised by humans, or assist in expressing the protein (expression enhancer) at higher yields than the native recombinant protein. Preferably the fusion partner will be both an immunological fusion partner and expression enhancing partner.

20 Fusion partners include protein D from *Haemophilus influenzae* and the non-structural protein from influenzae virus, NS1 (hemagglutinin). Another fusion partner is the protein known as LytA. Preferably the C terminal portion of the molecule is used. LytA is derived from *Streptococcus pneumoniae* which synthesize an N-acetyl-L-alanine amidase, amidase LytA, (coded by the *lytA* gene {Gene, 43 (1986) page 265-  
25 272}) an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LytA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E.coli* C-LytA expressing plasmids useful for

forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions.

- 5 Further preferred fragments include an isolated polypeptide comprising an amino acid sequence having at least 15, 20, 30, 40, 50 or 100 contiguous amino acids from the amino acid sequence of SEQ ID NO:2,4,6, or an isolated polypeptide comprising an amino acid sequence having at least 15, 20, 30, 40, 50 or 100 contiguous amino acids truncated or deleted from the amino acid sequence of SEQ ID NO:2,4,6.

10

Fragments of the polypeptides of the invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, these fragments may be employed as intermediates for producing the full-length polypeptides of the invention.

15

Particularly preferred are variants in which several, 5-10, 1-5, 1-3, 1-2 or 1 amino acids are substituted, deleted, or added in any combination.

20

The polypeptides, or immunogenic fragments, of the invention may be in the form of the "mature" protein or may be a part of a larger protein such as a precursor or a fusion protein. It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification such as multiple histidine residues, or an additional sequence for stability during recombinant production. Furthermore, addition of exogenous polypeptide or

25 lipid tail or polynucleotide sequences to increase the immunogenic potential of the final molecule is also considered.

In one aspect, the invention relates to genetically engineered soluble fusion proteins comprising a polypeptide of the present invention, or a fragment thereof, and various

The BASB040 polypeptides provided in SEQ ID NO:2,4,6 are the BASB040 polypeptides from *Neisseria meningitidis* strains ATCC13090 and H44/76.

The invention also provides an immunogenic fragment of a BASB040 polypeptide. that  
5 is, a contiguous portion of the BASB040 polypeptide which has the same or substantially  
the same immunogenic activity as the polypeptide comprising the amino acid sequence of  
SEQ ID NO:2,4,6. That is to say, the fragment (if necessary when coupled to a carrier) is  
capable of raising an immune response which recognises the BASB040 polypeptide.  
Such an immunogenic fragment may include, for example, the BASB040 polypeptide  
10 lacking an N-terminal leader sequence, and/or a transmembrane domain and/or a C-  
terminal anchor domain. In a preferred aspect the immunogenic fragment of BASB040  
according to the invention comprises substantially all of the extracellular domain of a  
polypeptide which has at least 85% identity, more preferably at least 90% identity, yet  
more preferably at least 95% identity, most preferably at least 97-99% identity, to that  
15 of SEQ ID NO:2,4,6 over the entire length of SEQ ID NO:2,4,6.

A fragment is a polypeptide having an amino acid sequence that is entirely the same as part  
but not all of any amino acid sequence of any polypeptide of the invention. As with  
BASB040 polypeptides, fragments may be "free-standing," or comprised within a larger  
20 polypeptide of which they form a part or region, most preferably as a single continuous  
region in a single larger polypeptide.

Preferred fragments include, for example, truncation polypeptides having a portion of an  
amino acid sequence of SEQ ID NO:2,4,6 or of variants thereof, such as a continuous series  
25 of residues that includes an amino- and/or carboxyl-terminal amino acid sequence.  
Degradation forms of the polypeptides of the invention produced by or in a host cell, are  
also preferred. Further preferred are fragments characterized by structural or functional  
attributes such as fragments that comprise alpha-helix and alpha-helix forming regions,  
beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-

The invention relates to BASB040 polypeptides and polynucleotides as described in greater detail below. In particular, the invention relates to polypeptides and polynucleotides of BASB040 of *Neisseria meningitidis*, which is related by amino acid sequence homology to *Neisseria meningitidis* D15 outer membrane protein. The invention relates especially to BASB040 having the nucleotide and amino acid sequences set out in SEQ ID NO:1,3,5 and SEQ ID NO:2,4,6 respectively. It is understood that sequences recited in the Sequence Listing below as "DNA" represent an exemplification of one embodiment of the invention, since those of ordinary skill will recognize that such sequences can be usefully employed in polynucleotides in general, including ribopolynucleotides.

### Polypeptides

In one aspect of the invention there are provided polypeptides of *Neisseria meningitidis* referred to herein as "BASB040" and "BASB040 polypeptides" as well as biologically, diagnostically, prophylactically, clinically or therapeutically useful variants thereof, and compositions comprising the same.

The present invention further provides for:

- (a) an isolated polypeptide which comprises an amino acid sequence which has at least 85% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, most preferably at least 97-99% or exact identity, to that of SEQ ID NO:2, 4, 6;
- (b) a polypeptide encoded by an isolated polynucleotide comprising a polynucleotide sequence which has at least 85% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, even more preferably at least 97-99% or exact identity to SEQ ID NO:1, 3, 5 over the entire length of SEQ ID NO:1, 3, 5 respectively; or
- (c) a polypeptide encoded by an isolated polynucleotide comprising a polynucleotide sequence encoding a polypeptide which has at least 85% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, even more preferably at least 97-99% or exact identity, to the amino acid sequence of SEQ ID NO:2, 4, 6;

crucial for immunity against meningococcal disease (Ross, S.C., Rosenthal P.J., Berberic, H.M., Densen, P. J. Infect. Dis. 155: 1266-1275, 1987).

5 The frequency of *Neisseria meningitidis* infections has risen dramatically in the past few decades. This has been attributed to the emergence of multiply antibiotic resistant strains and an increasing population of people with weakened immune systems. It is no longer uncommon to isolate *Neisseria meningitidis* strains that are resistant to some or all of the standard antibiotics. This phenomenon has created an unmet medical need and demand for new anti-microbial agents, vaccines, drug screening methods, and diagnostic tests for this  
10 organism.

#### SUMMARY OF THE INVENTION

The present invention relates to BASB040, in particular BASB040 polypeptides and  
15 BASB040 polynucleotides, recombinant materials and methods for their production. In another aspect, the invention relates to methods for using such polypeptides and polynucleotides, including prevention and treatment of microbial diseases, amongst others. In a further aspect, the invention relates to diagnostic assays for detecting diseases associated with microbial infections and conditions associated with such infections, such  
20 as assays for detecting expression or activity of BASB040 polynucleotides or polypeptides.

Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following  
25 descriptions and from reading the other parts of the present disclosure.

#### DESCRIPTION OF THE INVENTION



The polysaccharide vaccines are currently being improved by way of chemical conjugating them to carrier proteins (Lieberman, J.M., Chiu, S.S., Wong, V.K., et al. JAMA 275 : 1499-1503, 1996).

5

A serogroup B vaccine is not available, since the B capsular polysaccharide was found to be nonimmunogenic, most likely because it shares structural similarity to host components (Wyle, F.A., Artenstein, M.S., Brandt, M.L. et al. J. Infect. Dis. 126: 514-522, 1972; Finne, J.M., Leinonen, M., Mäkelä, P.M. Lancet ii: 355-357, 1983).

10

For many years efforts have been initiated and carried out to develop meningococcal outer membrane based vaccines (de Moraes, J.C., Perkins, B., Camargo, M.C. et al. Lancet 340: 1074-1078, 1992; Bjune, G., Hoiby, E.A. Gronnesby, J.K. et al. 338: 1093-1096, 1991). Such vaccines have demonstrated efficacies from 57% - 85% in older children (>4 years) and adolescents.

15

Many bacterial outer membrane components are present in these vaccines, such as PorA, PorB, Rmp, Opc, Opa, FrpB and the contribution of these components to the observed protection still needs further definition. Other bacterial outer membrane components have been defined by using animal or human antibodies to be potentially relevant to the induction of protective immunity, such as TbpB and NspA (Martin, D., Cadieux, N., Hamel, J., Brodeux, B.R., J. Exp. Med. 185: 1173-1183, 1997; Lissolo, L., Maître-Wilmotte, C., Dumas, p. et al., Inf. Immun. 63: 884-890, 1995). The mechanisms of protective immunity will involve antibody mediated bactericidal activity and opsonophagocytosis.

20

25

A bacteremia animal model has been used to combine all antibody mediated mechanisms (Saukkonen, K., Leinonen, M., Abdillahi, H. Poolman, J. T. Vaccine 7: 325-328, 1989). It is generally accepted that the late complement component mediated bactericidal mechanism is

**POLYPEPTIDES AND POLYNUCLEOTIDES "BASB040" FROM NEISSERIA  
MENINGITIDIS AND VACCINE COMPRISING SAID POLYPEPTIDES AND  
POLYNUCLEOTIDES**

**FIELD OF THE INVENTION**

- 5 This invention relates to polynucleotides, (herein referred to as "BASB040  
polynucleotide(s)"), polypeptides encoded by them (referred to herein as "BASB040" or  
"BASB040 polypeptide(s)"), recombinant materials and methods for their production. In  
another aspect, the invention relates to methods for using such polypeptides and  
polynucleotides, including vaccines against bacterial infections. In a further aspect, the  
10 invention relates to diagnostic assays for detecting infection of certain pathogens.

**BACKGROUND OF THE INVENTION**

- Neisseria meningitidis* (meningococcus) is a Gram-negative bacterium frequently isolated  
from the human upper respiratory tract. It occasionally causes invasive bacterial diseases  
15 such as bacteremia and meningitis. The incidence of meningococcal disease shows  
geographical seasonal and annual differences (Schwartz, B., Moore, P.S., Broome, C.V.;  
Clin. Microbiol. Rev. 2 (Supplement), S18-S24, 1989). Most disease in temperate countries  
is due to strains of serogroup B and varies in incidence from 1-10/100,000/year total  
population sometimes reaching higher values (Kaczmarek, E.B. (1997), Commun. Dis.  
20 Rep. Rev. 7: R55-9, 1995; Scholten, R.J.P.M., Bijlmer, H.A., Poolman, J.T. et al. Clin.  
Infect. Dis. 16: 237-246, 1993; Cruz, C., Pavez, G., Aguilar, E., et al. Epidemiol. Infect.  
105: 119-126, 1990).

- Epidemics dominated by serogroup A meningococci, mostly in central Africa, are  
25 encountered, sometimes reaching levels up to 1000/100.000/year (Schwartz, B., Moore,  
P.S., Broome, C.V. Clin. Microbiol. Rev. 2 (Supplement), S18-S24, 1989). Nearly all cases  
as a whole of meningococcal disease are caused by serogroup A, B, C, W-135 and Y  
meningococci and a tetravalent A, C, W-135, Y polysaccharide vaccine is available  
(Armand, J., Arminjon, F., Mynard, M.C., Lafaix, C., J. Biol. Stand. 10: 335-339, 1982).

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/31, C07K 14/22, 16/12, A61K 39/095, C12N 15/62, C12Q 1/68, G01N 33/566</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/34480</b> <b>(43) International Publication Date:</b> 15 June 2000 (15.06.00)
<b>(21) International Application Number:</b> PCT/EP99/09560 <b>(22) International Filing Date:</b> 2 December 1999 (02.12.99) <b>(30) Priority Data:</b> 9826886.5 7 December 1998 (07.12.98) GB <b>(71) Applicant (for all designated States except US):</b> SMITHKLINE BEECHAM BIOLOGICALS S.A. [BE/BE]; 89, rue de l'Institut, B-1330 Rixensart (BE). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> RUELLE, Jean-Louis [BE/BE]; SmithKline Beecham Biologicals s.a., 89, rue de l'Institut, B-1330 Rixensart (BE). <b>(74) Agent:</b> PRIVETT, Kathryn, Louise; SmithKline Beecham, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).	<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> POLYPEPTIDES AND POLYNUCLEOTIDES "BASB040" FROM NEISSERIA MENINGITIDIS AND VACCINE COMPRISING SAID POLYPEPTIDES AND POLYNUCLEOTIDES  <b>(57) Abstract</b>  The invention provides BASB040 polypeptides and polynucleotides from Neisseria meningitidis encoding BASB040 polypeptides and methods for producing such polypeptides by recombinant techniques. Also provided are diagnostic, prophylactic and therapeutic uses.  <div data-bbox="836 1386 1185 1585" style="text-align: center; border: 1px solid black; padding: 5px; margin: 20px auto; width: fit-content;">23 JUN 2000 INTERNATIONAL BUREAU</div>		

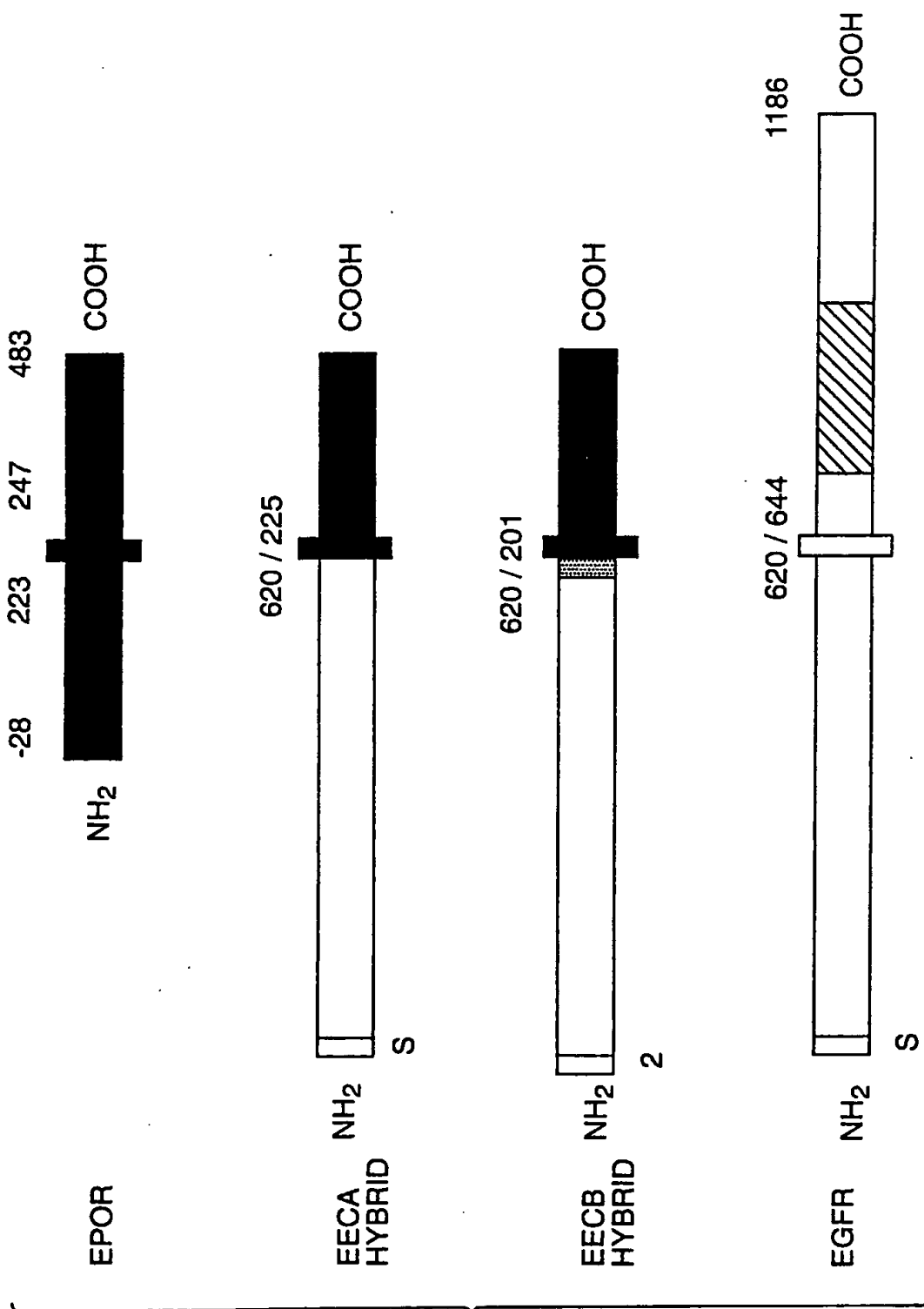


FIG. 1



FIG. 2A

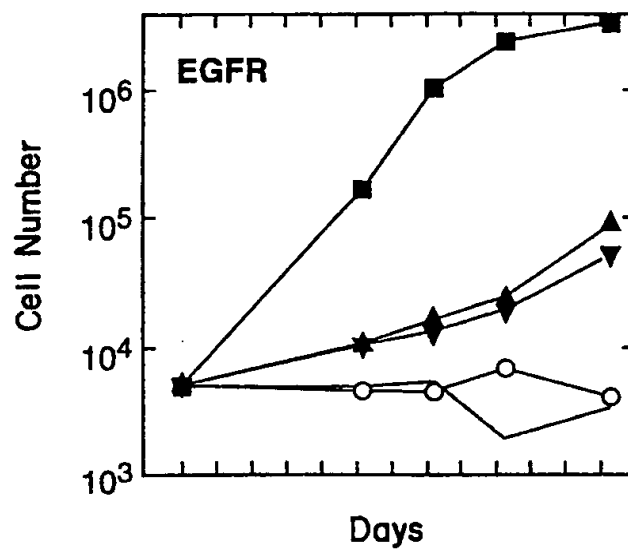


FIG. 2B

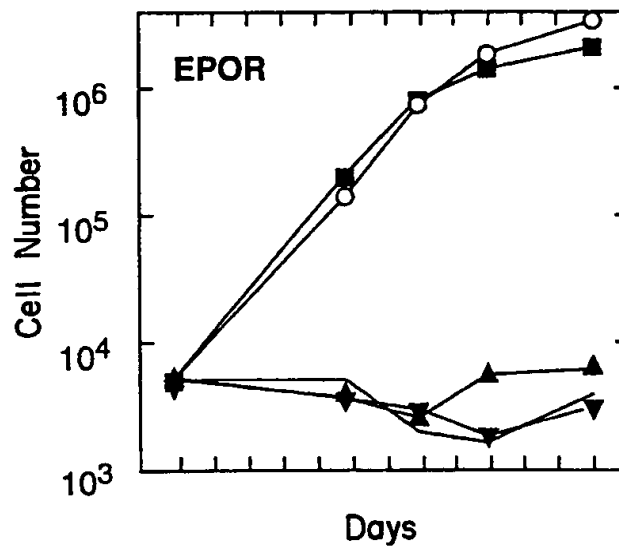






FIG. 2C

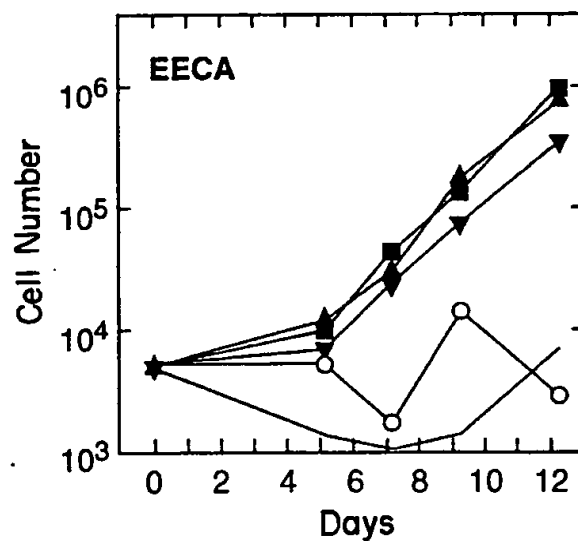


FIG. 2D

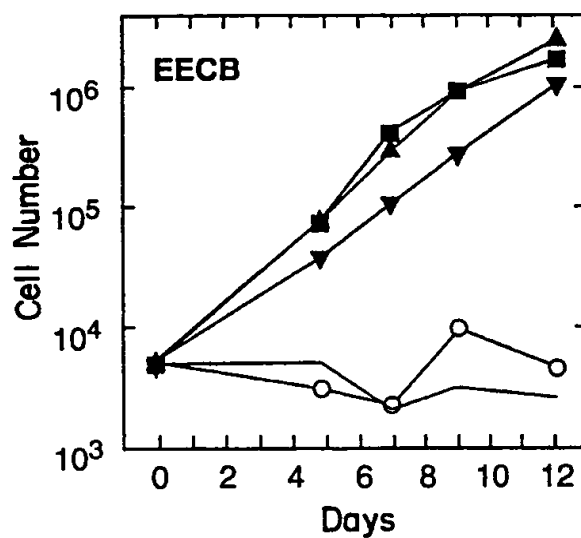
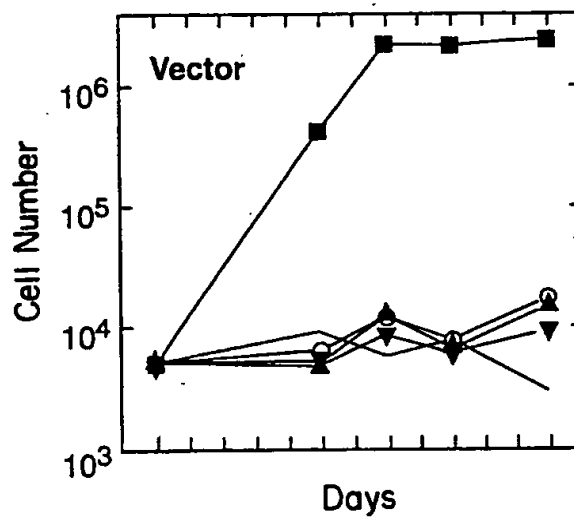


FIG. 2E





## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/06280

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/62 C07K15/00 C12N15/85 C12N5/10 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 533 006 (F. HOFFMANN-LA ROCHE & CO.) 24 March 1993 see claims see examples 12,13 ---	1,7,13, 19,25,31
X	SCIENCE, vol.252, no.5005, 26 April 1991, WASHINGTON DC, USA pages 561 - 563 H. YAN ET AL. 'Chimeric NGF-EGF receptors define domains responsible for neuronal differentiation.' cited in the application see abstract --- -/-	1,7,13, 19,37



Further documents are listed in the continuation of box C.



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Date of the actual completion of the international search

6 October 1994

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Name and mailing address of the ISA

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NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
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## INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol.84, no.8, April 1987, WASHINGTON DC, USA pages 2125 - 2129 O. BERNARD ET AL. 'High-affinity interleukin 2 binding by an oncogenic hybrid interleukin 2-epidermal growth factor receptor molecule.' see abstract see figure 1 ---	1,7,13, 19
A	EP,A,0 244 221 (GENENTECH INC.) 4 November 1987  see examples ---	1,7,13, 19,25, 31,37
A	NATURE, vol.324, no.6092, 6 November 1986, LONDON, GB pages 68 - 70 H. RIEDEL ET AL. 'A chimaeric receptor allows insulin to stimulate tyrosine kinase activity of epidermal growth factor receptor.' see abstract see figure 1 ---	1,7,13, 19,25, 31,37
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol.269, no.3, 21 January 1994, BALTIMORE MD, USA pages 1571 - 1574 R. PACIFICI ET AL. 'Hybrid tyrosine kinase/cytokine receptors transmit mitogenic signals in response to ligand.' see the whole document ---	1,4-7, 10-13, 16-19, 22-25,37
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol.91, no.1, 4 January 1994, WASHINGTON DC, USA pages 158 - 162 H. OHASHI ET AL. 'Ligand-induced activation of chimeric receptors between the erythropoietin receptor and receptor tyrosine kinases.' see abstract see figure 1 --- -/--	1,4-7, 10-13, 16-19,37



## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 94/06280

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol.269, no.8, 25 February 1994, BALTIMORE MD, USA pages 5976 - 5980 K. MARUYAMA ET AL. 'Proliferation and erythroid differentiation through the cytoplasmic domain of the erythropoietin receptor.' see abstract see figure 1</p> <p>-----</p>	<p>1,4-7, 10-13, 16-19,37</p>





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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/EP94/04227 <b>(22) International Filing Date:</b> 15 December 1994 (15.12.94)  <b>(30) Priority Data:</b> 9326253.3 23 December 1993 (23.12.93) GB  <b>(71) Applicant (for all designated States except US):</b> SMITHKLINE BEECHAM BIOLOGICALS (S.A.) [BE/BE]; 89, rue de l'Institut, B-1330 Rixensart (BE).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> MOMIN, Patricia, Marie [BE/BE]; SmithKline Beecham Biologicals (S.A.), 89, rue de l'Institut, B-1330 Rixensart (BE). GARCON, Nathalie, Marie-Joséphé [FR/FR]; SmithKline Beecham Biologicals (S.A.), 89, rue de l'Institut, B-1330 Rixensart (BE).  <b>(74) Agent:</b> DALTON, Marcus, Jonathan, William; SmithKline Beecham, Corporate Intellectual Property, SB House, Great West Road, Brentford, Middlesex TW8 9BD (GB).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> VACCINES		
<b>(57) Abstract</b>  The present invention provides vaccine compositions comprising an oil in water emulsion optionally with 3 De-O-acylated monophosphoryl lipid A and QS21. The vaccines' compositions are potent inducers of a range of immune responses.		



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- 1 -

### Vaccines

The present invention relates to novel vaccine formulations, to methods of their production and to their use in medicine. In particular, the present invention relates to an oil in water emulsion. Such emulsions comprise tocopherol, squalene, Tween 80, Span 85 and Lecithin and have useful adjuvant properties. Vaccines containing QS21, an Hplc purified non-toxic fraction derived from the bark of Quillaja Saponaria Molina, and/or 3 De-O-acylated monophosphoryl lipid A (3 D-MPL), together with such oil in water emulsions also form part of the invention.

10

3 De-O-acylated monophosphoryl lipid A is known from GB2220 211 (Ribi). Chemically it is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem Montana. A preferred form of 3 De-O-acylated monophosphoryl lipid A is disclosed in International Patent Application No. 92/116556.

15

QS21 is a Hplc purified non toxic fraction of a saponin from the bark of the South American tree Quillaja Saponaria Molina and its method of its production is disclosed (as QA21) in US patent No. 5,057,540.

20

Oil in water emulsions per se are known in the art, and have been suggested to be useful as adjuvant compositions (EPO 399843).

The present invention is based on the surprising discovery that an oil in water emulsion of the present invention, which unlike emulsions of the prior art contain tocopherol, as such or in combination with QS21 and/or 3 D-MPL enhance immune responses to a given antigen. Such enhancement available affords better immunological responses than hitherto before.

25

Additionally the oil in water emulsions of the present invention when formulated with 3 D-MPL and QS21 are preferential stimulators of IgG2a production and TH1 cell response. This is advantageous, because of the known implication of TH1 response in cell mediated response. Indeed in mice induction of IgG2a is correlated with such an immune response.

30

For example a vaccine formulation of the HIV antigen gp120 in such a combination results in a powerful synergistic induction of gp120 protein specific immune responses.

35



The observation that it is possible to induce strong cytolytic T lymphocyte responses is significant as these responses, in certain animal models have been shown to induce protection against disease.

5

The present inventors have shown that the combination of the adjuvants QS21 and 3D-MPL together with an oil in water emulsion with an antigen results in a powerful induction of CS protein specific CTL in the spleen. QS21 also enhances induction of CTL on its own, while 3D-MPL does not.

10

Induction of CTL is easily seen when the target antigen is synthesised intracellularly (e.g. in infections by viruses, intracellular bacteria, or in tumours), because peptides generated by proteolytic breakdown of the antigen can enter the appropriate processing pathway, leading to presentation in association with class I molecules on the cell membrane. However, in general, pre-formed soluble antigen does not reach

15

this processing and presentation pathway, and does not elicit class I restricted CTL. Therefore conventional non-living vaccines, while eliciting antibody and T helper responses, do not generally induce CTL mediated Immunity. The combination of the two adjuvants QS21 and 3D-MPL together with an oil in water emulsion can overcome this serious limitation of vaccines based on recombinant proteins, and induce a wider spectrum of immune responses.

20

CTL specific for CS protein have been shown to protect from malaria in mouse model systems (Romero et al. Nature 341:323 (1989)). In human trials where volunteers were immunised using irradiated sporozoites of *P. falciparum*, and shown to be protected against subsequent malaria challenge, induction of CTL specific for CS epitopes was demonstrated (Malik et al. Proc. Natl. Acad. Sci. USA 88:3300 (1991)).

25

The ability to induce CTL specific for an antigen administered as a recombinant molecules is relevant to malaria vaccine development, since the use of irradiated sporozoites would be impractical, on the grounds of production and the nature of the immune response.

30

RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of *P. falciparum* linked via four amino acids of the preS<sub>2</sub> portion of Hepatitis B surface antigen to the surface (S) antigen of hepatitis B virus. Its full structure is disclosed in co-pending International Patent Application No. PCT/EP92/02591, published under Number WO 93/10152 claiming priority from UK

35





patent application No.9124390.7. When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS,S.

- 5 In addition to human immunodeficiency virus and malaria vaccines, the ability to induce CTL responses would benefit vaccines against herpes simplex virus, cytomegalovirus, and generally all cases where the pathogen has an intracellular life stage.
- 10 Likewise, CTL specific for known tumour antigens could be induced by a combination of a recombinant tumour antigen and the two adjuvants. This would allow the development of anti cancer vaccines.

In certain systems, the combination of 3D-MPL and QS21 together with an oil in  
15 water emulsion have been able to synergistically enhance interferon  $\gamma$  production. The present inventors have demonstrated the potential of 3D-MPL and QS21 together with an oil in water emulsion by utilising a herpes simplex antigen known as gD<sub>2t</sub>. gD<sub>2t</sub> is a soluble truncated glycoprotein D from HSV-2 and is produced in CHO cells according to the methodology Berman *et al.* Science 222 524-527.

20 IFN- $\gamma$  secretion is associated with protective responses against intracellular pathogens, including parasites, bacteria and viruses. Activation of macrophages by IFN- $\gamma$  enhances intracellular killing of microbes and increases expression of Fc receptors. Direct cytotoxicity may also occur, especially in synergism with lymphotoxin (another  
25 product of TH1 cells). IFN- $\gamma$  is also both an inducer and a product of NK cells, which are major innate effectors of protection. TH1 type responses, either through IFN- $\gamma$  or other mechanisms, provide preferential help for IgG2a immunoglobulin isotypes.

Glycoprotein D is located on the viral envelope, and is also found in the cytoplasm of  
30 infected cells (Eisenberg R.J. *et al.* J. of Virol. 1980 35 428-435). It comprises 393 amino acids including a signal peptide and has a molecular weight of approximately 60kD. Of all the HSV envelope glycoproteins this is probably the best characterized (Cohen *et al.* J. Virology 60 157-166). *In vivo* it is known to play a central role in viral attachment to cell membranes. Moreover, glycoprotein D has been shown to be  
35 able to elicit neutralizing antibodies *in vivo* (Eing *et al.* J. Med Virology 127: 59-65). However, latent HSV2 virus can still be reactivated and induce recurrence of the disease despite the presence of high neutralizing antibodies titre in the patients sera. It



- 4 -

is therefore apparent that the ability to induce neutralizing antibody alone is insufficient to adequately control the disease.

5 In order to prevent recurrence of the disease, any vaccine will need to stimulate not only neutralizing antibody, but also cellular immunity mediated through T-cells, particularly cytotoxic T-cells.

10 In this instance the gD<sub>2t</sub> is HSV2 glycoprotein D of 308 amino acids which comprises amino acids 1 through 306 of the naturally occurring glycoprotein with the addition of Asparagine and Glutamine at the C terminal end of the truncated protein. This form of the protein includes the signal peptide which is cleaved to yield a mature 283 amino acid protein. The production of such a protein in Chinese Hamster ovary cells has been described in Genentech's European patent EP-B-139 417.

15 The mature truncated glycoprotein D (rgD<sub>2t</sub>) or equivalent proteins secreted from mammalian cells, is preferably used in the vaccine formulations of the present invention.

20 The formulations of the present invention are very effective in inducing protective immunity in a genital herpes model in guinea pigs. Even with very low doses of antigen (e.g. as low as 5 µg rgD<sub>2t</sub>) the formulations protect guinea pigs against primary infection and also stimulate specific neutralising antibody responses. The inventors, utilising formulation of the present invention, have also demonstrated Effector cell mediated responses of the TH1 type in mice.

25 Accordingly, in one preferred embodiment of the present invention provides a vaccine or pharmaceutical formulation comprising an antigen in conjunction with 3 De-O-acylated monophosphoryl lipid A, QS21 and an oil in water emulsion wherein the oil in water emulsion comprises a metabolisable oil, such as squalene, alpha tocopherol and  
30 tween 80. Such a formulation is suitable for a broad range of monovalent or polyvalent vaccines. Additionally the oil in water emulsion may contain span 85. A preferred form of 3 De-O-acylated monophosphoryl lipid A is disclosed in International patent application published under No. 92116556 - SmithKline Beecham Biologicals s.a.

35 The oil in water emulsion may be utilised on its own or with other adjuvants or immuno-stimulants and therefore an important embodiment of the invention is an oil in



water formulation comprising squalene or another metabolisable oil, alpha tocopherol, and tween 80. The oil in water emulsion may also contain span 85 and/or Lecithin.

5 Preferably the vaccine formulations will contain an antigen or antigenic composition capable of eliciting an immune response against a human or animal pathogen, which antigen or antigenic composition is derived from HIV-1, (such as gp120 or gp160), any of Feline Immunodeficiency virus, human or animal herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus ((esp Human)(such as gB or derivatives thereof), Varicella Zoster  
10 Virus (such as gpI, II or III), or from a hepatitis virus such as hepatitis B virus for example Hepatitis B Surface antigen or a derivative thereof, hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as Respiratory Syncytial virus, human papilloma virus or Influenza virus, or derived from bacterial pathogens such as Salmonella, Neisseria, Borrelia (for example OspA or OspB or  
15 derivatives thereof), or Chlamydia, or Bordetella for example P.69, PT and FHA, or derived from parasites such as plasmodium or Toxoplasma.

The formulations may also contain an anti-tumour antigen and be useful for immunotherapeutically treating cancers.

20 In an immunotherapeutic animal model for B cell lymphoma, where BCL-1 mouse lymphoma cells are administered intraperitoneally to Balb/c mice on day 0, and mice are vaccinated on days 3, 10 and 20 with the BCL-1 Idiotypic formulation SB62/MPL/QS21 stands out as the most potent, both with respect to antibody titers, and with respect to survival (the only group with 100% survival). Similarly the ability  
25 of this formulation to stimulate cytotoxic T lymphocytes to the antigens included make them a good candidate for formulation of cancer antigens (eg melanoma antigens MAGE-1 and MAGE-3 for immunotherapy of tumors by active vaccination).

30 The formulation may also be useful for utilising with herpetic light particles such as described in International Patent Application No. PCT/GB92/00824 and, International Patent Application No. PCT/GB92/00179.

35 Derivatives of Hepatitis B Surface antigen are well known in the art and include, inter alia, those PreS<sub>1</sub>, PreS<sub>2</sub> S antigens set forth described in European Patent applications EP-A-414 374; EP-A-0304 578, and EP 198-474. In one preferred aspect the vaccine formulation of the invention comprises the HIV-1 antigen, gp120, especially when



expressed in CHO cells. In a further embodiment, the vaccine formulation of the invention comprises gD<sub>2t</sub> as hereinabove defined.

5 In a further aspect of the present invention there is provided a vaccine as herein described for use in medicine.

The ratio of QS21 : 3D-MPL will typically be in the order of 1 : 10 to 10 : 1; preferably 1 : 5 to 5 : 1 and often substantially 1 : 1. The preferred range for optimal synergy is 2.5:1 to 1:1 3D MPL: QS21. Typically for human administration QS21 and  
10 3D MPL will be present in a vaccine in the range 1 µg - 100 µg, preferably 10 µg - 50 µg per dose. Typically the oil in water will comprise from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% tween 80. Preferably the ratio of squalene: alpha tocopherol is equal or less than 1 as this provides a more stable emulsion. Span 85 may also be present at a level of 1%. In some cases it may be  
15 advantageous that the vaccines of the present invention will further contain a stabiliser.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A.  
20 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

25 The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 µg of protein, preferably 2-100 µg, most preferably 4-40 µg. An  
30 optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced.

35 The formulations of the present invention maybe used for both prophylatic and therapeutic purposes.





Accordingly in one aspect, the invention provides a method of treatment comprising administering an effective amount of a vaccine of the present invention to a patient.

The following examples illustrate the invention.

5

### Examples

**Example 1** Vaccine formulation comprising the gp120 antigen of HIV-1.

10 The two adjuvant formulations were made each comprising the following oil in water emulsion component.

SB26: 5% squalene 5% tocopherol 0.4% tween 80; the particle size was 500 nm size

SB62: 5% Squalene 5% tocopherol 2.0% tween 80; the particle size was 180 nm

15

1(a) Preparation of emulsion SB62 (2 fold concentrate)

Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100 ml two fold concentrate emulsion 5g of DL alpha tocopherol  
20 and 5ml of squalene are vortexed to mix thoroughly. 90ml of PBS/Tween solution is added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M1105 microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

25 1(b) Preparation of emulsion SB26

This emulsion was prepared in an analogous manner utilising 0.4% tween 80.

1(c) Other emulsions as depicted in Table 1 were made in an analogous manner. These  
30 are tested in the experiments as detailed in the following examples.

1(d) Preparation of gp 120 QS21/3D MPL oil in water formulation.

To the emulsion of 1 a) or b) or c) an equal volume of twice concentrated rgp120  
35 (either 20µg or 100µg) was added and mixed. This was combined with 50µg/ml of 3D-MPL and 20µg/ml of QS21 to give the final formulation. Buffer was sed according to salt content and pH.



Table 3 shows the effectiveness of SB26, utilising gp120 from HIV and 50µg/ml 3D MPL (MPL) and 20µg/ml of QS21. The results show the geometric mean titre (GMT) after the second (P11) and third (P111) inoculations as well as cell mediated responses (CMI) to lymphocyte proliferation and  $\gamma$  interferon production.

5

## Example 2

Introduction: Evaluation of an HIV gp 120 emulsion system

- 10 In this experiment, four emulsions are compared [SB26, SB 62, SB40, SB61]. The influence of each formulation's component (antigen, emulsion, 3D- MPL, QS21) is evaluated.

### 2(b) Groups of animals utilised

15

There are 22 groups of 5 animals each group received a different vaccine formulation.

- gr 1-4: gp 120 (10µg) / no emuls ± [3D-MPL, QS21]
- gr 5-9: gp 120 (10µg) / SB26 ± [3D-MPL, QS21]
- 20 - gr 10: no antigen / SB26 + [3D-MPL, QS21]
- gr 11-12: gp 120 (10µg) / SB62 ± [3D-MPL, QS21]
- gr 13-16: gp 120 (10µg) / SB40 ± [3D-MPL, QS21]
- gr 17-20: gp 120 (10µg) / SB61 ± [3D-MPL, QS21]
- gr 21-22: gp 120 (5µg) / SB26 ± [3D-MPL, QS21]

25

- Assays: - antibody titers to gp 120W61D and isotype analysis (all groups)

### 2(c) Immunization and bleeding schedule

- 30 - animals were immunized with gp 120W61D, formulated in different o/w emulsions in the presence of 5µg 3D-MPL and 5µg QS21 per dose. Negative controls received the equivalent formulations without any antigen.

- animals were immunized subcutaneously at day 0 and 14. Each injection dose  
35 was administered in a 100µl volume.

- blood samples were obtained before Immunization (day 0) and after Immunization on days 14 (post I), 21 and 28 (7 and 14d. post II).



2(d) **Analysis of the serological response:**

- the 14 days post I and post II serological response was evaluated in a direct  
5 ELISA assay to gp 120W61D.
- the 14 days post II response was also characterized regarding the isotypes of  
gp 120W61D specific antibodies induced in mice after immunization.

10 **3 RESULTS AND DISCUSSION:**

The results are depicted on Table 2

a) **Comparison of emulsions in the presence or absence of 3D-MPL/QS21:**

15

- Addition of emulsions SB26, SB40 or SB62 to the antigen induces higher  
antibody titers; In the absence of immunostimulants, the gp 120 specific antibodies  
are essentially IgG1.

20

- Addition of immunostimulants 3D-MPL and QS21 induces a huge serological  
response and a shift of antibodies from IgG1 type to IgG2a/IgG2b. This correlated  
with cell mediated immunity.

The preferred combination is [SB26 + MPL + QS21] .

25

c) **gp120/SB26 formulation:**

No significant difference in serological response is observed between group 8 and  
group 9; addition of the gp 120 before or after the other components of the  
formulation.

30

d) **Antigen dose:**

Both 5 and 10 µg of gp 120 formulated in SB26 induce high serological response  
(groups 5-8 and 21-22) .

35 **Example 3 HSV rgD<sub>2</sub>t formulation**

In analogous manner to that set forth in Example 1a) formulation comprising the  
herpes simplex antigen rgD<sub>2</sub>t was made and used to vaccinate guinea pigs. Such



formulation induced protection against both recurrent and initial disease in the guinea pig model.

#### Example 4

#### 5 Screening of adjuvants for induction of protective anti lymphoma responses using idiotype as immunogen.

Therapeutic vaccination of Balb/c mice with idiotype from BCL1 lymphoma cells.

- 10 A review of the BALB/C B-cell lymphoma model is discussed by Yefenoh et al. Current opinions Immunobiology 1993 5:740-744.

- 15 Groups of 10 mice are injected (ip) with  $10^4$  tumor cells at day 0, and vaccinated with 100 µg of KLH- coupled immunoglobulin directed against BCL 1 epitoped (ratio of KLH/g: 1/1), in different adjuvant formulations at days 3, 10, 20 (sc immunization in the back). Level of serum antibodies to KLH and to idiotype, as well as mouse death are monitored.

Formulations tested:

group#	adjuvant	MPL: 10µg
1	none (no antigen)	QS21: 10µg
2	none	
3	Freund	
4	Alum	
5	Alum/MPL	
6	Alum/MPL/QS21	
7	QS21	
8	MPL/QS21	
9	SB62MPL	
10	SB62/MPL/QS21	

20

groups 12-15: different adjuvants without antigen

Formulations 8, 9, 10, behaved consistently better as compared to the others.

- 25 Formulation 10 stands out as the most potent, both with respect to antibody titers, and with respect to survival (the only group with 100% survival).





**EXAMPLE 5 Various formulations of RTS,S****a) Evaluated in monkeys**

5

RTS,S is described in International patent application no. WO93/10152 and was formulated for vaccination of Rhesus monkeys. Five animals were in each group:

- |    |           |  |
|----|-----------|--|
| 10 | Group I   | RTS,S, 3D-MPL(50 $\mu$ ), AL(OH) <sub>3</sub>      |
|    | Group II  | RTS,S, QS21(20 $\mu$ ), AL(OH) <sub>3</sub>        |
|    | Group III | RTS,S, 3D-MPL(50 $\mu$ ), QS21(20 $\mu$ )          |
|    | Group IV  | RTS,S, 3D-MPL(50 $\mu$ ), QS21 AL(OH) <sub>3</sub> |
|    | Group V   | RTS,S, 3D-MPL(10 $\mu$ ), QS21 AL(OH) <sub>3</sub> |
|    | Group VI  | RTS,S, 3D-MPL(50 $\mu$ ), QS21 SB60                |

15

The animals were inoculated and bled at 14 days post first immunisation and 12 days post second immunisation and tested for Anti hepatitis B surface antigen immunoglobulin. As can be seen from figure 1, animals receiving RTS,S, in SB60 had antibody titres almost six fold higher than any other group.

20

**b) Various formulations of RTS,S - Evaluated in mice**

7 groups of animals received the following formulations

- |    |         |                                  |
|----|---------|----------------------------------|
| 25 | Group 1 | RTS,S SB62                       |
|    | Group 2 | RTS,S QS21 3D-MPL                |
|    | Group 3 | RTS,S QS21 3D-MPL SB26           |
|    | Group 4 | RTS,S 3D-MPL A1(OH) <sub>3</sub> |
|    | Group 5 | RTS,S A1(OH) <sub>3</sub>        |
| 30 | Group 6 | Plain                            |
|    | Group 7 | Negative control                 |

(RTS,S - 5 $\mu$ g/dose, 3 D-MPL 5 $\mu$ g/dose QS21 5 $\mu$ g/dose)

- 35 The animals were inoculated and bled at 15 days post first immunisation and at day 7 and 15 post second immunisation and assayed for anti HBSAg antibody subtype. As can be seen from figure 2, the emulsion SB62 when formulated with QS21 and 3D-



MPL enhances preferentially and in a synergistic fashion the IgG2a antibody response while SB 62 alone or 3 D- MPL / QS21 induce a poor IgG2a response.

EXAMPLE 6: Evaluation of different B burgdorferi OspA formulations

5

6.1 Evaluation of different formulations of B burgdorferi ZS7 Osp A lipoproteins.

OspA lipoprotein for B burgdorferi is described in European Patent Application 0418 827 Max Plank et al.

10

The following formulations were tested in balb/c mice

1. OspA + Al(OH)<sub>3</sub>
2. OspA + Al(OH)<sub>3</sub> + 3D-MPL (10μ)
- 15 3. OspA + Al(OH)<sub>3</sub> + 3D-MPL (30μ)
4. OspA + Al(OH)<sub>3</sub> + 3D-MPL (10μ) + QS21 (5μ)
5. OspA + Al(OH)<sub>3</sub> + 3D-MPL (30μ) + QS21 (15μ)
6. OspA + SB60 + 3D-MPL (10μ) + QS21 (5μ)
7. OspA + SB60 + 3D-MPL (30μ) + QS21 (15μ)

20

and antibody titres and sub types studied seven days following a first inoculation and seven days post second inoculation (inoculations were at day 0, and 14).

25

The results depicted graphically in figures 3 and 4 and show that the formulations of the present invention induce high levels of antibodies and these are preferentially of the IgG2a subtype.

EXAMPLE 7:

30 a) HSV-2 ICP 27

Female Balb/c mice were immunized on day 0 and day 14 in the hind foot-pads with various formulations of NS1-ICP27. Each injection contained 5 μg of NS1-ICP27 and combinations of SB26 oil-in-water emulsion, QS21 (10 μg) and MPL (25 μg).

35

Popliteal lymphnode cells were obtained on day 28 and stimulated in vitro with syngeneic P815 cells transfected with the ICP27 gene. The cultures were then tested for specific cytolytic activity on P815 target cells transfected with ICP27 and P815 ICP27 negative controls.



- 13 -

Specific lysis results at different effector:target (E:T) ratios for different immunization groups were as follows:

ICP 27 (5 $\mu$ g)

5	E:T	P815	P815 transfected with ICP 27 clone 121
	100:1	-1	0
	30:1	-2	-3
	10:1	3	0
	3:1	1	0
10	1:1	2	2
	0.3:1	2	2

ICP 27 (5 $\mu$ g) + MPL (25 $\mu$ g)

	E:T	P815	P815 transfected with ICP 27 clone 121
15	100:1	5	7
	30:1	2	2
	10:1	1	2
	3:1	-1	-1
	1:1	-2	-2
20	0.3:1	-4	-1

ICP 27 (5 $\mu$ g) + QS21 (10 $\mu$ g)

	E:T	P815	P815 transfected with ICP 27 clone 121
	100:1	4	17
25	30:1	5	10
	10:1	3	7
	3:1	4	5
	1:1	3	5
	0.3:1	0	1
30			

ICP 27 (5 $\mu$ g) + SB26

	E:T	P815	P815 transfected with ICP 27 clone 121
	100:1	5	20
	30:1	1	19
35	10:1	2	12
	3:1	-2	7
	1:1	1	5
	0.3:1	1	2



- 14 -

ICP 27 (5µg) + MPL (25µg) + QS21 (10µg)

E:T P815 P815 transfected with ICP 27 clone 121

100:1 4 13  
 5 30:1 5 12  
 10:1 4 17  
 3:1 1 3  
 1:1 0 3  
 0.3:1 -1 -2

10

ICP 27 (5µg) + MPL (25µg) + QS21 (10µg) + SB26

E:T P815 P815 transfectés avec ICP27 clone 121

100:1 2 20  
 30:1 0 17  
 15 10:1 3 19  
 3:1 3 8  
 1:1 1 6  
 0.3:1 2 3

20 Low ICP27 specific % lysis was obtained in immunization groups:

ICP 27 (5µg) + QS21 (10µg)

ICP 27 (5µg) + SB26

ICP 27 (5µg) + MPL (25µg) + QS21 (10µg)

25 ICP 27 (5µg) + MPL (25µg) + QS21 (10µg) + SB26

while

ICP 27 (5µg)

30 ICP 27 (5µg) + MPL (25µg)

were negative.

Thus these data show induction of CTL by recombinant NS1-ICP27 in  
 oil-in-water emulsion alone or with QS21 and MPL; or with QS21.

35

b) Groups of 5 Balb/c mice were vaccinated in the footpad with the different  
 vaccines (NS1-ICP27/NS1-ICP27 MPL + QS21/NS1-ICP27 SB26 = MPL and QS21/  
 adjuvant alone). One dose contained 10 µg NS1-ICP27, 10 µg MPL and 10µg QS21.





Two vaccinations were given at days 0 and 7. Mice were challenged at day 14 with  $5.2 \times 10^3$  TCID<sub>50</sub> of HSV2 strain MS. The appearance of zosteriform lesions and deaths were recorded until day 14 post challenge.

- 5 ICP27 of HSV2 was expressed in E coli as a fusion protein with NS1 fragment of influenza virus. The protective efficacy of the purified recombinant protein was evaluated in the murine zosteriform model, in combination with MPL QS21 formulations. Balb/c mice given two vaccinations with NS1-ICP27 combined either with MPL + QS21 or with an oil in water emulsion (SB26) + MPL and QS21 were
- 10 completely protected against disease (no zosteriform lesions) and death following HSV2 wild type challenge. In contrast, protection was not observed in the mice vaccinated either with NS1-ICP27 alone or with NS1-ICP27 combined with SB26 without MPL and QS21.



**Table 1**  
**Vehicles two fold concentrated**

Emulsions SB	Tocopherol %	Squalene %	Tween 80 %	Span 85 %	Lecithin %	Size
26	5	5	0.4	0	0	500 nm 90-100% 800 nm 10-0%
26.1	5	5	0.4	0	0.1	500 nm
63	5	5	0.6	0	0	500 nm
64	5	5	0.8	0	0	500 nm
61	5	5	1	0	0	250-300 nm
62	5	5	2	0	0	180 nm
40	5	5	0.4	1	0	500 nm 80-100% 800 nm 20-0%
40.1	5	5	0.4	1	0.1	500 nm
65	5	5	0.4	1.5	0	500 nm
65	5	5	0.4	2	0	500 nm



Table 2  
HIV gp 120W61D / MOUSE IMMUNOGENICITY (94243) / BALB / C (F.P.)

GROUPS	IMMUNOGEN (dose)/FORMULATION	ELISA TITERS (7 days PII)	% IgG1	%IgG2a	% IgG2b
1	gp120 10µg	494	100	0	0
2	gp120 10µg + 3D-MPL 5µg	4164	54	15	32
3	gp120 10µg + QS21 5µg	21515	89	4	8
4	gp120 10µg + 3D-MPL + QS21	52749	22	60	18
5	gp120 10µg / SB26	12205	94	2	4
6	gp120 10µg / SB26 + 3D-MPL	87388	31	42	27
7	gp120 10µg / SB26 + QS21	51020	73	15	13
8	gp120 10µg / SB26 + 3D-MPL + QS21	178169	23	57	21
9	SB26 + 3D-MPL + QS21 / gp120 10µg	185704	22	60	19
11	gp120 10µg / SB62	10348	92	8	0
12	gp120 10µg / SB62 + 3D-MPL + QS21	21739	54	37	9
13	gp120 10µg / SB40	36320	90	7	4
14	gp120 10µg / SB40 + 3D-MPL	285219	31	44	25
15	gp120 10µg / SB40 + QS21	48953	78	15	7
16	gp120 10µg / SB40 + 3D-MPL + QS21	209217	14	67	18
17	gp120 10µg / SB61	<50	-	-	-
18	gp120 10µg / SB61 + 3D-MPL	77515	31	50	19
19	gp120 10µg / SB61 + QS21	40737	74	13	13
20	gp120 10µg / SB61 + 3D-MPL + QS21	59673	29	57	14
21	gp120 5µg / SB26	25089	99	0	1
22	gp120 5µg / SB26 + 3D-MPL + QS21	242736	18	61	21

ELISA titers to gp 120 W61D: geomean of 5 individual titers, calculated by LINEST



Table 3  
3D-MPL based formulations: HIV protect Monkey studies

Read-Out	GMT ELisa W61 D <sub>1</sub>		GMT Neut. MN		DTH in vivo	CMI in vitro		
	P11	P111	P11	P111		LP	IL-2	γIFN
gp120 (100 µg)/ o/w + MPL + QS21	60523	93410	1:500	>1:3200		+	ND	+
gp120 (20 µg)/ o/w + MPL + QS21	52026	50150	1:500	1:2400		+	ND	+
"Historical" gp120 (100 µg)/ o/w + MPL in guinea pigs		20064						





**Claims**

1. A vaccine composition comprising an antigen and/or antigenic composition, QS21, 3 De-O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein the oil in water emulsion has the following composition: a metabolisable oil, such as squalene, alpha tocopherol and tween 80.
2. A vaccine as claimed in claim 1 wherein the ratio of QS21:3D-MPL is from 1:10 to 10:1.
3. A vaccine composition as claimed in claim 1 or 2 capable of invoking a cytolytic T cell response in a mammal to the antigen or antigenic composition.
4. A vaccine composition as claimed in any of claims 1 to 3 capable of stimulating interferon  $\gamma$  production.
5. A vaccine composition as claimed in any of claims 1 to 4 wherein the ratio of QS21:3D-MPL is from 1:1 to 1:2.5.
6. A vaccine composition as claimed herein comprising an antigen or antigenic composition derived from any of Human Immunodeficiency Virus, Feline Immunodeficiency Virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A,B,C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma.
7. A vaccine as claimed in any of claim 1 to 5 wherein the antigen is a tumour antigen.
8. Use of composition as defined in any of claims 1 to 5 for the manufacture of a vaccine for the prophylactic treatment of viral, bacterial, or parasitic infections.
9. Use of composition as defined in any of claims 1 to 5 for the manufacture of a vaccine for the immunotherapeutic treatment of viral, bacterial, parasitic infections or cancer.
10. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.



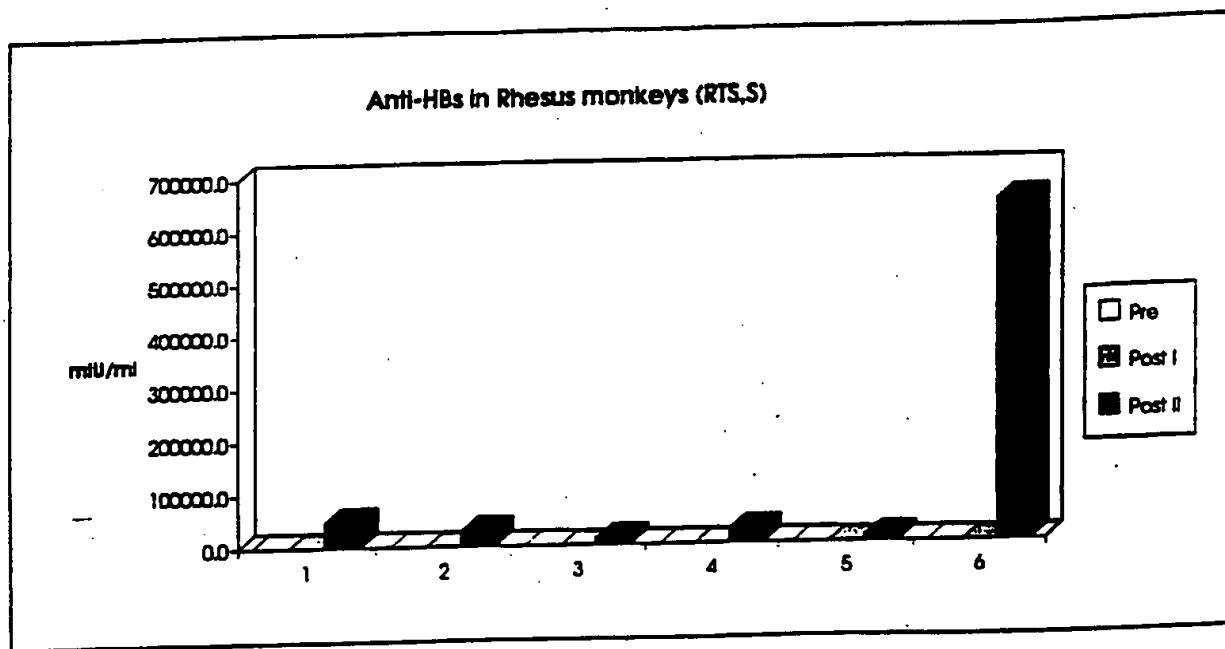
11. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.

12. A process for making a vaccine composition according to claims 1 to 5 comprising admixing QS21, 3D-MPL and the oil in water emulsion as defined in claim 1 with an antigen or antigenic composition.

13. A vaccine composition comprising an antigen or antigenic composition in association with an oil in water emulsion which emulsion comprises: a metabolisable oil, alpha tocopherol, and tween 80.



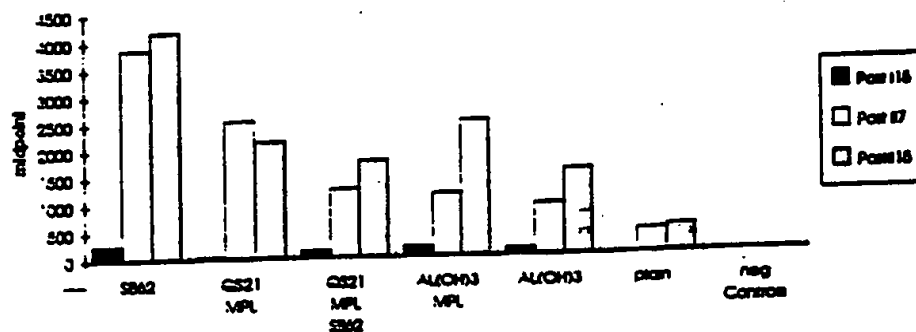
1/4

Figure 1

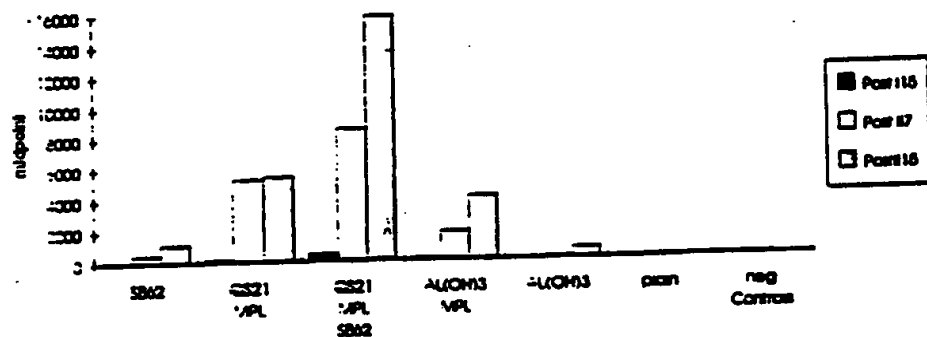


ANTIGEN HbsAg		IgG1			IgG2a			IgG2b		
GROUP	FORMULATION	Post 115	Post 17	Post 18	Post 115	Post 17	Post 18	Post 115	Post 17	Post 18
1	SB62	278	3861	4171	44	479	1134	61	700	700
2	GS21 MPL	33	2533	2144	178	5361	5464	138	2238	1168
3	GS21 MPL SB62	130	1248	1774	498	8581	16804	371	5187	3888
4	AL(OH)3 MPL	187	1134	2301	129	1832	4089	249	2821	1441
5	AL(OH)3	130	934	1842	13	128	484	288	192	951
6	plain	4	426	498	6	98	67	8	228	183
7	neg Control	5	5	5	5	5	5	5	5	5

anti HbsAg IgG1 pool



anti HbsAg IgG2a pool



anti HbsAg IgG2b pool



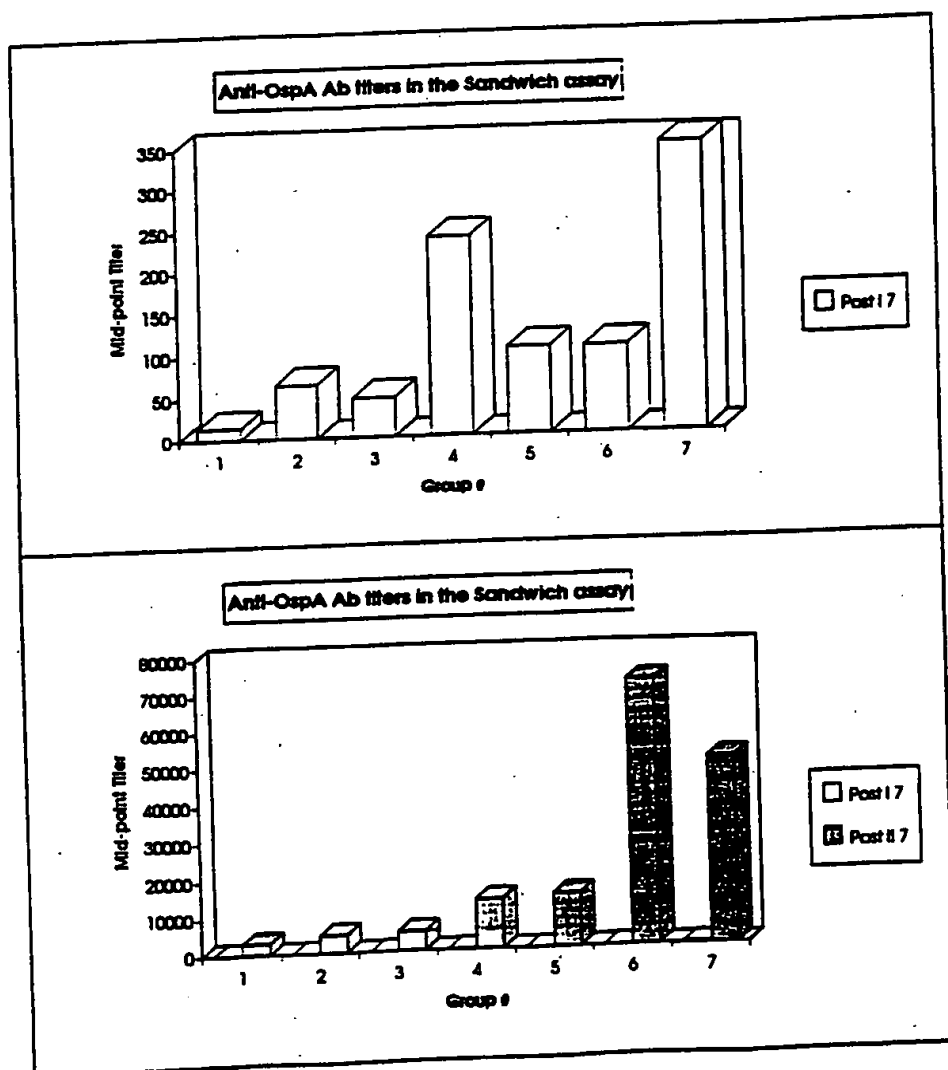




3/4

Anti-OspA Abs titers (igt) after immunization of Balb/C mice  
with different formulations of Lipoprotein OspA

Figure 3

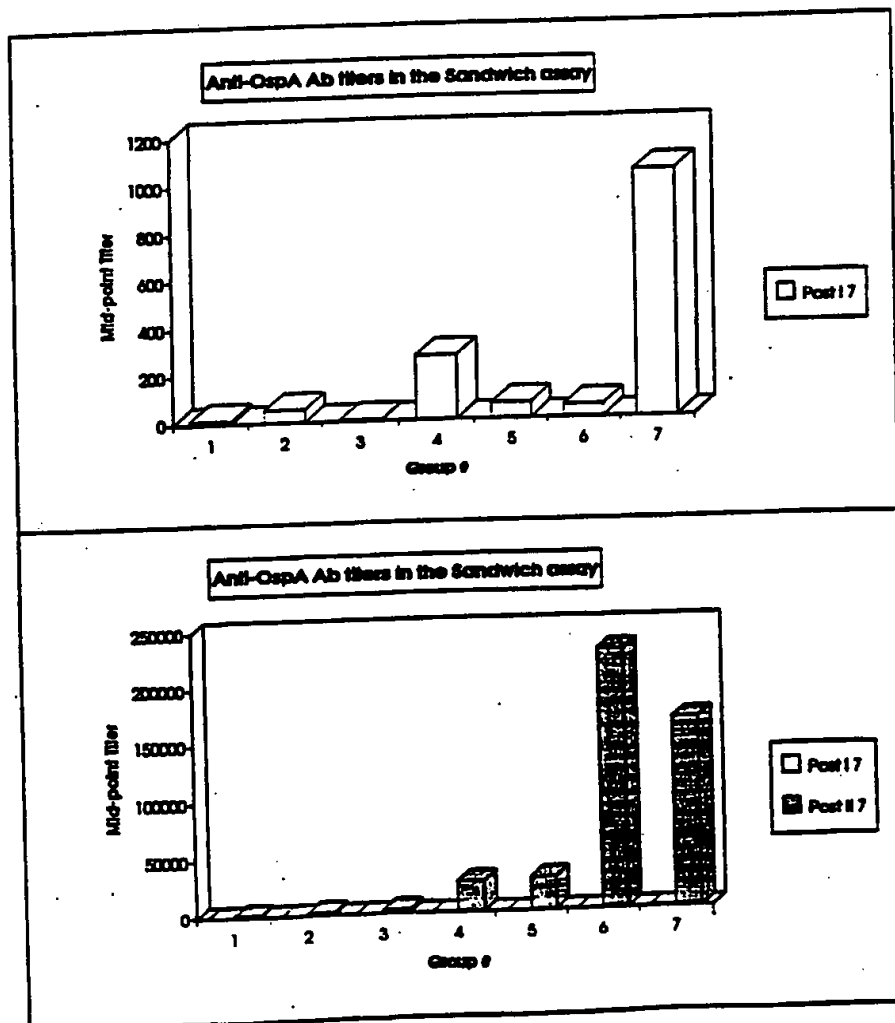




4 / 4

Anti-OspA Abs titers (IgG2a) after immunization of Balb/C mice  
with different formulations of Lipoprotein OspA

Figure 4





# INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/EP 94/04227

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K39/39

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 16556 (SMITHKLINE BEECHAM BIOLOGICALS) 1 October 1992 cited in the application see the whole document ---	1-13
Y	EP,A,0 399 843 (CHIRON CORPORATION) 28 November 1990 cited in the application see the whole document ---	1,6-13
Y	EP,A,0 382 271 (AKZO) 16 August 1990 see the whole document ---	1,6-13
Y	WO,A,88 09336 (CAMBRIDGE BIOSCIENCE CORPORATION) 1 December 1988 cited in the application see the whole document ---	1,6-13
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "A" document member of the same patent family

Date of the actual completion of the international search

22 March 1995

Date of mailing of the international search report

30.03.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Moreau, J



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 94/04227

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	<p>WO,A,94 00153 (SMITHKLINE BEECHAM BIOLOGICALS) 6 January 1994 see the whole document -----</p>	1-13





# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 94/04227

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 10-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 94/04227

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9216556	01-10-92	AP-A- 368	28-10-94
		AU-B- 654970	01-12-94
		AU-A- 8510991	28-04-92
		BR-A- 9107294	14-06-94
		CN-A- 1064891	30-09-92
		CZ-A- 9301957	18-05-94
		WO-A- 9206113	16-04-92
		EP-A- 0550485	14-07-93
		EP-A- 0644201	22-03-95
		HU-A- 67011	30-01-95
		JP-T- 6501151	10-02-94
EP-A-0399843	28-11-90	AT-T- 108327	15-07-94
		CA-A- 2017507	25-11-90
		DE-D- 69010574	18-08-94
		DE-T- 69010574	27-10-94
		ES-T- 2033626	16-10-94
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		AU-B- 633043	21-01-93
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		CA-A- 2008856	04-08-90
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		JP-A- 2250835	08-10-90
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		CA-A- 1331443	16-08-94
		DE-D- 3852761	23-02-95
		EP-A- 0362279	11-04-90
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		AU-B- 4326493	24-01-94
		CA-A- 2138996	06-01-94
		CA-A- 2138997	06-01-94



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

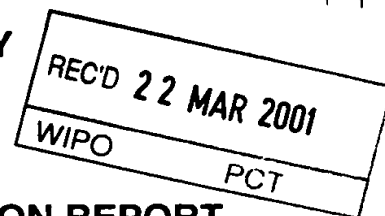
PCT/EP 94/04227

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9400153		CN-A- 1086142	04-05-94
		CN-A- 1092812	28-09-94
		WO-A- 9400575	06-01-94
		NO-A- 945003	23-12-94
		SI-A- 9300335	31-12-93
-----			



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)



Applicant's or agent's file reference KP/BM45339	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/EP99/09560	International filing date (day/month/year) 02/12/1999	Priority date (day/month/year) 07/12/1998
International Patent Classification (IPC) or national classification and IPC C12N15/31		
Applicant SMITHKLINE BEECHAM BIOLOGICALS S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.


2. This REPORT consists of a total of 6 sheets, including this cover sheet.

- ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand  30/06/2000	Date of completion of this report  20.03.2001
Name and mailing address of the international preliminary examining authority:   European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Kalsner, I  Telephone No. +49 89 2399 8708







**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP99/09560

**I. Basis of the report**

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).):*

**Description, pages:**

1-64 as originally filed

**Claims, No.:**

1-27 as originally filed

**Drawings, sheets:**

1/15-15/15 as originally filed

**Sequence listing part of the description, pages:**

1-11, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP99/09560

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 27 with respect to industrial applicability.

because:

- ☒ the said international application, or the said claims Nos. 27 relate to the following subject matter which does not require an international preliminary examination (*specify*):  
**see separate sheet**
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**



# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP99/09560

---

## 1. Statement

Novelty (N)	Yes:	Claims	1-27
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-23, 25-27
Industrial applicability (IA)	Yes:	Claims	
	No:	Claims	1-23, 25-27

## 2. Citations and explanations **see separate sheet**



**Ad Section III: Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

Claim 24 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. In this respect the following should be noted:

For the assessment of this claim on the question whether it is industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of this claim (Article 34(4)(a)(i) PCT).

**Ad Section V: Reasoned statement with regard to novelty, inventive step or industrial applicability**

**1) Novelty and inventive step**

- 1.1) The present application relates to polynucleotides isolated from *Neisseria meningitidis* and encoding a protein having 21% identity in a 623 amino acid overlap with the *Neisseria meningitidis* D15 outer membrane protein. The DNA sequence was derived from a database containing genomic DNA sequences of *N. meningitidis* strain ATCC13090.

The polypeptides and polynucleotides claimed in **claims 1-15** formally meet the requirements of Art. 33(2) PCT as the sequences have not been published in the prior art. Hence, **claims 16-27**, which relate to subject-matter dependent on claims 1-15 also meet the requirements of Art. 33(2) PCT.

- 1.2) **Claims 1-27** do not meet the requirements of Art. 33(3)(4) PCT for the following reasons.





**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

---

International application No. PCT/EP99/09560

The present application lacks any evidence that the claimed nucleic acid sequence (derived from a genomic library) actually encodes a functional protein, let alone a protein with antigenic properties which could be used in a vaccine composition as claimed.

Cloning of a gene the translated protein of which shows similarity to a known protein is considered routine laboratory praxis. In the present application no technical effect, such as e.g. antigenicity or protection against infection, etc., of the cloned gene is shown.

The applicant is reminded that demonstrated function of a newly identified protein or nucleic acid sequence is a prerequisite to the final assessment of inventive step and industrial applicability.

Thus, inventive step and industrial applicability can neither be acknowledged for the claims relating to the nucleic or amino acid sequences nor for the vaccine compositions comprising such sequences (**claims 1-27**).



by fax and post

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:

GIDDINGS, Peter John  
SmithKline Beecham  
Corporate Intellectual Property  
Two New Horizons Court  
Brentford  
Middlesex TW8 9EP  
GRANDE BRETAGNE

RECEIVED

15 MAR 2001

NEW HORIZONS COURT

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT

(PCT Rule 71.1)

FAX NO: +44 20 8975 6177

Date of mailing  
(day/month/year) 20.03.2001

Applicant's or agent's file reference  
KP/BM45339

## IMPORTANT NOTIFICATION

International application No.  
PCT/EP99/09560

International filing date (day/month/year)  
02/12/1999

Priority date (day/month/year)  
07/12/1998

Applicant

SMITHKLINE BEECHAM BIOLOGICALS S.A. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

## 4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/

European Patent Office  
D-80298 Munich  
Tel. +49 89 2399 - 0 Tx: 523656 epmu d  
Fax: +49 89 2399 - 4465

Authorized officer

Büchler, S

Tel. +49 89 2399-8090







## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference <b>KP/BM45339</b>		<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/PEA/416)	
International application No. <b>PCT/EP99/09560</b>		International filing date (day/month/year) <b>02/12/1999</b>	Priority date (day/month/year) <b>07/12/1998</b>
International Patent Classification (IPC) or national classification and IPC <b>C12N15/31</b>			
Applicant <b>SMITHKLINE BEECHAM BIOLOGICALS S.A. et al.</b>			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 6 sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of sheets.</p>			
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> <li>I <input checked="" type="checkbox"/> Basis of the report</li> <li>II <input type="checkbox"/> Priority</li> <li>III <input checked="" type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</li> <li>IV <input type="checkbox"/> Lack of unity of invention</li> <li>V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</li> <li>VI <input type="checkbox"/> Certain documents cited</li> <li>VII <input type="checkbox"/> Certain defects in the international application</li> <li>VIII <input type="checkbox"/> Certain observations on the international application</li> </ul>			
Date of submission of the demand <b>30/06/2000</b>		Date of completion of this report <b>20.03.2001</b>	
Name and mailing address of the international preliminary examining authority:  <b>European Patent Office</b> <b>0-80298 Munich</b> Tel. +49 89 2399 - 0 Tx: 523655 epmu d Fax +49 89 2399 - 4465		Authorized officer <b>Kaisner, I</b> Telephone No. +49 89 2399 8708 	



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP99/09560

**I. Basis of the report**

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*  
Description, pages:

1-64 as originally filed

**Claims, No.:**

1-27 as originally filed

**Drawings, sheets:**

1/15-15/15 as originally filed

**Sequence listing part of the description, pages:**

1-11, as originally filed

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:





**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**International application No. **PCT/EP99/09560**

- ☐ the description, pages:  
☐ the claims, Nos.:  
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.  
☒ claims Nos. 27 with respect to industrial applicability.

because:

- ☒ the said international application, or the said claims Nos. 27 relate to the following subject matter which does not require an international preliminary examination (*specify*):  
**see separate sheet**

- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

- ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- ☐ the written form has not been furnished or does not comply with the standard.  
☐ the computer readable form has not been furnished or does not comply with the standard.

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**International application No. PCT/EP99/09560

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**1. Statement**

Novelty (N)	Yes:	Claims 1-27
	No:	Claims
Inventive step (IS)	Yes:	Claims
	No:	Claims 1-23, 25-27
Industrial applicability (IA)	Yes:	Claims
	No:	Claims 1-23, 25-27

**2. Citations and explanations  
see separate sheet**



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/EP99/09560

**Ad Section III: N n-establishment of opini n with regard to novelty, inventiv  
step and industrial applicability**

Claim 24 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. In this respect the following should be noted:

For the assessment of this claim on the question whether it is industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of this claim (Article 34(4)(a)(i) PCT).

**Ad Section V: Reasoned statement with regard to novelty, inventive step or  
industrial applicability****1) Novelty and inventive step**

- 1.1) The present application relates to polynucleotides isolated from *Neisseria meningitidis* and encoding a protein having 21% identity in a 623 amino acid overlap with the *Neisseria meningitidis* D15 outer membrane protein. The DNA sequence was derived from a database containing genomic DNA sequences of *N. meningitidis* strain ATCC13090.

The polypeptides and polynucleotides claimed in **claims 1-15** formally meet the requirements of Art. 33(2) PCT as the sequences have not been published in the prior art. Hence, **claims 16-27**, which relate to subject-matter dependent on **claims 1-15** also meet the requirements of Art. 33(2) PCT.

- 1.2) **Claims 1-27** do not meet the requirements of Art. 33(3)(4) PCT for the following reasons.



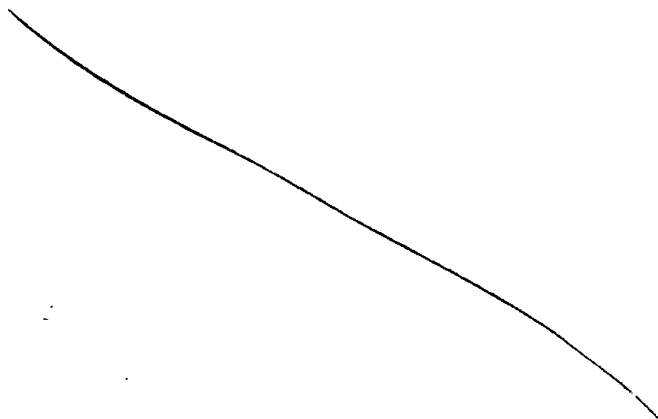
**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**International application No. **PCT/EP99/09560**

The present application lacks any evidence that the claimed nucleic acid sequence (derived from a genomic library) actually encodes a functional protein, let alone a protein with antigenic properties which could be used in a vaccine composition as claimed.

Cloning of a gene the translated protein of which shows similarity to a known protein is considered routine laboratory praxis. In the present application no technical effect, such as e.g. antigenicity or protection against infection, etc., of the cloned gene is shown.

The applicant is reminded that demonstrated function of a newly identified protein or nucleic acid sequence is a prerequisite to the final assessment of inventive step and industrial applicability.

Thus, inventive step and industrial applicability can neither be acknowledged for the claims relating to the nucleic or amino acid sequences nor for the vaccine compositions comprising such sequences (**claims 1-27**).





# INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/EP 99/09560

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/31 C07K14/22 C07K16/12 A61K39/095 C12N15/62  
C12Q1/68 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N A61K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. ARMAND ET AL.: "TETRAVALENT MENINGOCOCCAL POLYSACCHARIDE VACCINE GROUPS A, C, Y, W 135: CLINICAL AND SEOLOGICAL EVALUATION." JOURNAL OF BIOLOGICAL STANDARDIZATION, vol. 10, 1982, pages 335-339, XP000884367 cited in the application the whole document — —/—	

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "B" document member of the same patent family

Date of the actual completion of the international search

27 March 2000

Date of mailing of the international search report

04/04/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fac. (+31-70) 340-3016

Authorized officer

Hix, R

# INTERNATIONAL SEARCH REPORT

Int. Patent Application No  
PCT/EP 99/09560

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>J.M. LIEBERMAN ET AL.: "Safety and immunogenicity of a Serogroups A/C <i>Neisseria meningitidis</i> Oligosaccharide-protein conjugate vaccine in young children." THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, vol. 275, no. 19, 15 May 1996 (1996-05-15), pages 1499-1503, XP000884379 cited in the application the whole document</p>	
A	<p>F.A. WYLE ET AL.: "IMMUNOLOGIC RESPONSE OF MAN TO GROUP B MENINGOCOCCAL POLYSACCHARIDE VACCINES" JOURNAL OF INFECTIOUS DISEASES, vol. 126, no. 5, November 1972 (1972-11), pages 514-522, XP000884373 cited in the application the whole document</p>	
A	<p>D. MARTIN ET AL.: "Highly conserved <i>Neisseria meningitidis</i> surface protein confers protection against experimental infection." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 185, no. 7, 7 April 1997 (1997-04-07), pages 1173-1183, XP000884332 cited in the application the whole document</p>	
A	<p>L. LISSOLO ET AL.: "Evaluation of transferrin-binding protein 2 within the transferrin-binding protein complex as a potential antigen for future meningococcal vaccines." JOURNAL OF INFECTION AND IMMUNITY, vol. 63, no. 3, March 1995 (1995-03), pages 884-890, XP000877152 cited in the application the whole document</p>	

# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>KP/BM45339</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/EP 99/ 09560</b>	International filing date (day/month/year) <b>02/12/1999</b>	(Earliest) Priority Date (day/month/year) <b>07/12/1998</b>
Applicant  <b>SMITHKLINE BEECHAM BIOLOGICALS S.A. et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

**1. Basis of the report**

a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☒ contained in the international application in written form.

☒ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ Certain claims were found unsearchable (See Box I).

3. ☐ Unity of invention is lacking (see Box II).

**4. With regard to the title,**

☐ the text is approved as submitted by the applicant.

☒ the text has been established by this Authority to read as follows:

**POLYPEPTIDES AND POLYNUCLEOTIDES "BASB040" FROM NEISSERIA MENINGITIDIS AND VACCINE COMPRISING SAID POLYPEPTIDES AND POLYNUCLEOTIDES**

**5. With regard to the abstract,**

☐ the text is approved as submitted by the applicant.

☒ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

**6. The figure of the drawings to be published with the abstract is Figure No.**

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

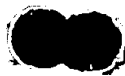
☐ because this figure better characterizes the invention.

☐ None of the figures.



## Box III TEXT OF THE ABSTRACT (Continuation of Item 5 of the first sheet)

The invention provides BASB040 polypeptides and polynucleotides from *Neisseria meningitidis* encoding BASB040 polypeptides and methods for producing such polypeptides by recombinant techniques.  
Also provided are diagnostic, prophylactic and therapeutic uses.



## INTERNATIONAL SEARCH REPORT

International Application No.

P 99/09560

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7. C12N15/31 C07K14/22 C07K16/12 A61K39/095 C12N15/62  
C12Q1/68 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N A61K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. ARMAND ET AL.: "TETRAVALENT MENINGOCOCCAL POLYSACCHARIDE VACCINE GROUPS A, C, Y, W 135: CLINICAL AND SEOLOGICAL EVALUATION." JOURNAL OF BIOLOGICAL STANDARDIZATION, vol. 10, 1982, pages 335-339, XP000884367 cited in the application the whole document --- -/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A" document member of the same patent family

Date of the actual completion of the international search

27 March 2000

Date of mailing of the international search report

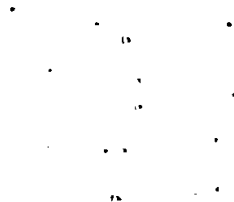
04/04/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5618 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3018

Authorized officer

Hix, R





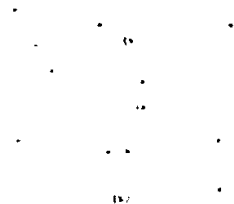
## INTERNATIONAL SEARCH REPORT

International Application No

P 99/09560

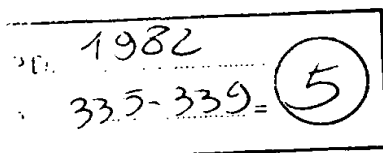
## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>J.M. LIEBERMAN ET AL.: "Safety and immunogenicity of a Serogroups A/C <i>Neisseria meningitidis</i> Oligosaccharide-protein conjugate vaccine in young children." THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, vol. 275, no. 19, 15 May 1996 (1996-05-15), pages 1499-1503, XP000884379 cited in the application the whole document</p> <p>---</p>	
A	<p>F.A. WYLE ET AL.: "IMMUNOLOGIC RESPONSE OF MAN TO GROUP B MENINGOCOCCAL POLYSACCHARIDE VACCINES" JOURNAL OF INFECTIOUS DISEASES, vol. 126, no. 5, November 1972 (1972-11), pages 514-522, XP000884373 cited in the application the whole document</p> <p>---</p>	
A	<p>D. MARTIN ET AL.: "Highly conserved <i>Neisseria meningitidis</i> surface protein confers protection against experimental infection." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 185, no. 7, 7 April 1997 (1997-04-07), pages 1173-1183, XP000884332 cited in the application the whole document</p> <p>---</p>	
A	<p>L. LISSOLO ET AL.: "Evaluation of transferrin-binding protein 2 within the transferrin-binding protein complex as a potential antigen for future meningococcal vaccines." JOURNAL OF INFECTION AND IMMUNITY, vol. 63, no. 3, March 1995 (1995-03), pages 884-890, XP000877152 cited in the application the whole document</p> <p>-----</p>	



XP-000884367

*Journal of Biological Standardization* (1982) 10, 335-339



**Tetravalent meningococcal  
polysaccharide vaccine groups A, C,  
Y, W 135: clinical and serological  
evaluation\***

*J. Armand†, F. Arminjon†,  
M. C. Mynard† and C. Lafaix‡*

A tetravalent meningococcal polysaccharide vaccine—groups A, C, Y, W 135—has been prepared for innocuity and immunogenicity testing in adult volunteers. In this preliminary study, the new formulation appeared safe and immunogenic.

**INTRODUCTION**

Although most of the isolations of *Nisseria meningitidis* of groups Y and W 135 are apparently from asymptomatic carriers, groups Y and W 135 cause a substantial number of acute symptomatic infections: septicemia and meningitis (Smilack, 1974; Koppes, Ellenbogen & Gebbart, 1977; Amico & Myerowitz, 1978; Kleiman, Reynolds, Steinfeld, Allen & Smith, 1978; Hersh, Gold & Lepow, 1979; McLeod, Griffiss & Brandt, 1979; Galaid *et al.*, 1980). In order to broaden the protection afforded by meningococcal vaccine, groups Y and W 135 polysaccharide components were added to groups A and C components.

The objective of this study was to evaluate the clinical acceptability of and the serological responses to this tetravalent formulation.

\* Received for publication 24 June 1982.

† Institut Merieux, 69260 Charbonnières Les Bains, France.

‡ Centre Hospitalier Intercommunal,—94 190 Villeneuve St Georges, France.

## MATERIALS AND METHODS

*Vaccine*

Capsular polysaccharides were prepared according to the method of Gorschlich, Rey, Etienne, Triaou & Cujetanovic (1972) and met the W.H.O. requirements (W.H.O., 1976, 1981). Quality controls are summarized in Table 1.

TABLE 1. Results of quality control tests performed on individual bulk polysaccharides and final vaccine

	Results				
	A polysaccharide	C polysaccharide	Y polysaccharide	W 135 polysaccharide	Final vaccine
Percentage polysaccharide eluted before $K_d$ 0.5	84.90	91.35	94.16	94.96	
Nucleic acids (% w/w)	0.16	0.35	0.29	0.27	
Protein (% w/w)	<0.10	<0.10	0.16	0.60	
Total nitrogen (% w/w)	—	—	2.46	2.17	
Sialic acid (% w/w)	—	81.16	53.04	57.60	
O Acetyl (mmol $g^{-1}$ )	2.12	1.58	0.57	0.39	
Phosphorus (% w/w)	8.25	—	0.13	<0.10	
Moisture (% w/w)	6.31	9.31	12.55	17.50	1.13
Rabbit pyrogen test	S	S	S	S	S
Innocuity	—	—	—	—	S
Sterility	—	—	—	—	S
Identity	S	S	S	S	S
Serological specificity	S	S	—	—	—

S, Satisfactory.

*Clinical studies*

The study group was composed of 24 healthy adult volunteer subjects (6 male and 18 female), aged 21–54 years (mean age of 31 years) and included employees and staff of a university hospital. Each received 0.5 ml of vaccine containing 50  $\mu$ g of each of the four polysaccharides, subcutaneously. None of the volunteers had previously received a meningococcal polysaccharide vaccine.

Each subject was monitored for local and systemic reactions for four days. Blood samples were collected prior to immunization and at four weeks after immunization. Bactericidal antibodies were measured using a procedure developed by the Bureau of Biologics (Food and Drug Administration, Bureau of Biologics, 1977).

## RESULTS

Neither systemic reactions nor temperatures above 37°C were observed. Mild local

# TETRAVALENT MENINGOCOCCAL POLYSACCHARIDE VACCINE

reactions at the injection site were reported but none persisted longer than 72 h (Table 2).

The results of bactericidal antibody tests indicated that out of 24 immunized individuals, 23 (95.8%), 24 (100%), 22 (91.7%) and 23 (95.8%) had fourfold or greater increases, respectively, in antibodies to groups A, C, Y and W 135. Table 3 shows, in particular, that 100% of seronegatives seroconverted after immunization.

TABLE 2. Local reactions after subcutaneous inoculation of a tetravalent meningococcal vaccine

Symptom duration (h)	Redness	Induration	Pain
24	4/24	1/24	8/24
48	5/24	4/24	7/24
72	1/24	2/24	0/24
Totals	10/24	7/24	15/24

TABLE 3. Meningococcal bactericidal antibody titres following immunization with a tetravalent meningococcal polysaccharide vaccine, groups A, C, Y, W 135

Meningococcal vaccine components	Initial seronegatives Titre <4			Initial seropositives Titre ≥4		
	Fourfold increase no./total*	%	g.m.c.†	Fourfold increase no./total*	%	g.m.c.†
Group A	3/3	100	(<4) 813	20/21	95	(56) 1573
Group C	12/12	100	(<4) 1932	12/12	100	(85) 1932
Group Y	9/9	100	(<4) 1392	13/15	87	(281) 2700
Group W 135	9/9	100	(<4) 1105	14/15	93	(67) 2962

\* Number showing rise/total number tested.

† Geometric mean titres, pre-immunization mean titres in parentheses.

## DISCUSSION

Several clinical trials in normal subjects have shown the immunogenicity of the individual polysaccharides of group Y and W 135 (Farquhar, Hankins, DeSanctis, DeMeio & Metzgar, 1978; Tiesjema & Beuvery, 1977), or the immunogenicity of group Y polysaccharide in combination with groups A and C polysaccharides (Farquhar, Hankins, DeSanctis, DeMeio & Metzgar, 1978). This study demonstrates very acceptable tolerance of this new vaccine composition and very good immunogenicity. After immunization subjects reached significant titres of bactericidal antibodies. All

J. ARMAND ET AL.

seronegatives seroconverted to the four components of the vaccine. Of the seropositives 95, 100, 87 and 93% achieved a fourfold or greater rise, respectively, to the A, C, Y and W 135 components. These results suggest an independent response to meningococcal polysaccharides. It remains necessary to study the persistence of antibodies and the immunogenicity of this combination in infants older than two years of age.

#### Acknowledgements

We thank Mrs Michèle Mignon for technical assistance and Nicole Gibelin for statistical work.

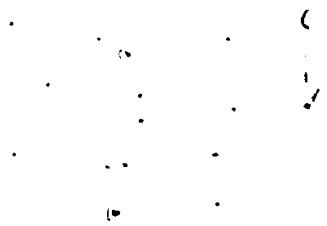
While this manuscript was in preparation, we learnt of similar work being done by Hankins, Gwaltney, Hendley, Farquhar & Samuelson (1982).

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# TETRAVALENT MENINGOCOCCAL POLYSACCHARIDE VACCINE

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# PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

# PCT

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL SEARCH REPORT  
OR THE DECLARATION

(PCT Rule 44.1)

To:

SMITHKLINE BEECHAM  
Corporate Intellectual Property  
Attn. Privett, Kathryn Louise  
Two New Horizons Court  
Brentford  
Middlesex TW8 9EP  
UNITED KINGDOM

Date of mailing  
(day/month/year)

04/04/2000

Applicant's or agent's file reference

KP/BM45339

**FOR FURTHER ACTION**

See paragraphs 1 and 4 below

International application No.

PCT/EP 99/09560

International filing date  
(day/month/year)

02/12/1999

Applicant

SMITHKLINE BEECHAM BIOLOGICALS S.A. et al.

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

**Filing of amendments and statement under Article 19:**

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

**When?** The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

**Where?** Directly to the International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland  
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the International application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the International application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority



European Patent Office, P.B. 5618 Patentlaan 2  
NL-2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3016

Authorized officer

Sandra De Jong-van Dam



These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

## INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

### What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

### When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

### Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

### How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

### What documents must/may accompany the amendments?

**Letter (Section 205(b)):**

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.



The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:  
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:  
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:  
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or  
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:  
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

#### "Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

#### Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

#### Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.



## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>KP/BM45339</b>		<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/EP 99/09560</b>	International filing date (day/month/year) <b>02/12/1999</b>	(Earliest) Priority Date (day/month/year) <b>07/12/1998</b>	
Applicant <b>SMITHKLINE BEECHAM BIOLOGICALS S.A. et al.</b>			

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

## 1. Basis of the report

- a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☒ contained in the international application in written form.

☒ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. ☐ Certain claims were found unsearchable (See Box I).

3. ☐ Unity of invention is lacking (see Box II).

## 4. With regard to the title,

☐ the text is approved as submitted by the applicant.

☒ the text has been established by this Authority to read as follows:

**POLYPEPTIDES AND POLYNUCLEOTIDES "BASB040" FROM NEISSERIA MENINGITIDIS AND VACCINE COMPRISING SAID POLYPEPTIDES AND POLYNUCLEOTIDES**

## 5. With regard to the abstract,

☐ the text is approved as submitted by the applicant.

☒ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

## 6. The figure of the drawings to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☐ None of the figures.





**Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)**

The invention provides BASB040 polypeptides and polynucleotides from *Neisseria meningitidis* encoding BASB040 polypeptides and methods for producing such polypeptides by recombinant techniques.  
Also provided are diagnostic, prophylactic and therapeutic uses.



## INTERNATIONAL SEARCH REPORT

International Application No.

T/EP 99/09560

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/31 C07K14/22 C07K16/12 A61K39/095 C12N15/62  
C12Q1/68 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N A61K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>J. ARMAND ET AL.: "TETRAVALENT MENINGOCOCCAL POLYSACCHARIDE VACCINE GROUPS A, C, Y, W 135: CLINICAL AND SEOLOGICAL EVALUATION." JOURNAL OF BIOLOGICAL STANDARDIZATION, vol. 10, 1982, pages 335-339, XP000884367 cited in the application the whole document</p> <p style="text-align: center;">-/-</p>	

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"S" document member of the same patent family

Date of the actual completion of the international search

27 March 2000

Date of mailing of the international search report

04/04/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3016

Authorized officer

Hix, R



## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 99/09560

C.(Continuation) DOCUMENTS CITED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>J.M. LIEBERMAN ET AL.: "Safety and immunogenicity of a Serogroups A/C <i>Neisseria meningitidis</i> Oligosaccharide-protein conjugate vaccine in young children."  THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION,  vol. 275, no. 19,  15 May 1996 (1996-05-15), pages 1499-1503,  XP000884379  cited in the application  the whole document</p>	
A	<p>F.A. WYLE ET AL.: "IMMUNOLOGIC RESPONSE OF MAN TO GROUP B MENINGOCOCCAL POLYSACCHARIDE VACCINES"  JOURNAL OF INFECTIOUS DISEASES,  vol. 126, no. 5, November 1972 (1972-11),  pages 514-522, XP000884373  cited in the application  the whole document</p>	
A	<p>D. MARTIN ET AL.: "Highly conserved <i>Neisseria meningitidis</i> surface protein confers protection against experimental infection."  JOURNAL OF EXPERIMENTAL MEDICINE,  vol. 185, no. 7,  7 April 1997 (1997-04-07), pages  1173-1183, XP000884332  cited in the application  the whole document</p>	
A	<p>L. LISSOLO ET AL.: "Evaluation of transferrin-binding protein 2 within the transferrin-binding protein complex as a potential antigen for future meningococcal vaccines."  JOURNAL OF INFECTION AND IMMUNITY,  vol. 63, no. 3, March 1995 (1995-03),  pages 884-890, XP000877152  cited in the application  the whole document</p>	



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 7 :</b> C12N 15/31, C07K 14/22, 16/12, A61K 39/095, C12N 15/62, C12Q 1/68, G01N 33/566	<b>A1</b>	<b>(11) International Publication Number:</b> WO 00/34480 <b>(43) International Publication Date:</b> 15 June 2000 (15.06.00)
<b>(21) International Application Number:</b> PCT/EP99/09560 <b>(22) International Filing Date:</b> 2 December 1999 (02.12.99) <b>(30) Priority Data:</b> 9826886.5 7 December 1998 (07.12.98) GB <b>(71) Applicant (for all designated States except US):</b> SMITHKLINE BEECHAM BIOLOGICALS S.A. [BE/BE]; 89, rue de l'Institut, B-1330 Rixensart (BE). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> RUELLE, Jean-Louis [BE/BE]; SmithKline Beecham Biologicals s.a., 89, rue de l'Institut, B-1330 Rixensart (BE). <b>(74) Agent:</b> PRIVETT, Kathryn, Louise; SmithKline Beecham, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> POLYPEPTIDES AND POLYNUCLEOTIDES "BASB040" FROM NEISSERIA MENINGITIDIS AND VACCINE COM-PRISING SAID POLYPEPTIDES AND POLYNUCLEOTIDES  <b>(57) Abstract</b>  The invention provides BASB040 polypeptides and polynucleotides from Neisseria meningitidis encoding BASB040 polypeptides and methods for producing such polypeptides by recombinant techniques. Also provided are diagnostic, prophylactic and therapeutic uses.		



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CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		



# INTERNATIONAL SEARCH REPORT

Application No  
**PCT/EP 99/09560**

**A. CLASSIFICATION F. SUBJECT MATTER**  
**IPC 7 C12N15/31 C07K14/22 C07K16/12 A61K39/095 C12N15/62**  
**C12Q1/68 G01N33/566**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

**IPC 7 C07K C12N A61K C12Q G01N**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<b>A</b>	<p><b>J. ARMAND ET AL.: "TETRAVALENT MENINGOCOCCAL POLYSACCHARIDE VACCINE GROUPS A, C, Y, W 135: CLINICAL AND SEOLOGICAL EVALUATION."</b>  <b>JOURNAL OF BIOLOGICAL STANDARDIZATION,</b>  <b>vol. 10, 1982, pages 335-339, XP000884367</b>  <b>cited in the application</b>  <b>the whole document</b></p> <p style="text-align: center;">— —/—</p>	

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

**\* Special categories of cited documents :**

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "O" document referring to an oral disclosure, use, exhibition or other means
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- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

**27 March 2000**

Date of mailing of the international search report

**04/04/2000**

Name and mailing address of the ISA  
 European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax (+31-70) 340-3018

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**Hix, R**



# INTERNATIONAL SEARCH REPORT

Application No.  
PCT/EP 99/09560

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>J.M. LIEBERMAN ET AL.: "Safety and immunogenicity of a Serogroups A/C <i>Neisseria meningitidis</i> Oligosaccharide-protein conjugate vaccine in young children." THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, vol. 275, no. 19, 15 May 1996 (1996-05-15), pages 1499-1503, XP000884379 cited in the application the whole document</p>	
A	<p>F.A. WYLE ET AL.: "IMMUNOLOGIC RESPONSE OF MAN TO GROUP B MENINGOCOCCAL POLYSACCHARIDE VACCINES" JOURNAL OF INFECTIOUS DISEASES, vol. 126, no. 5, November 1972 (1972-11), pages 514-522, XP000884373 cited in the application the whole document</p>	
A	<p>D. MARTIN ET AL.: "Highly conserved <i>Neisseria meningitidis</i> surface protein confers protection against experimental infection." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 185, no. 7, 7 April 1997 (1997-04-07), pages 1173-1183, XP000884332 cited in the application the whole document</p>	
A	<p>L. LISSOLO ET AL.: "Evaluation of transferrin-binding protein 2 within the transferrin-binding protein complex as a potential antigen for future meningococcal vaccines." JOURNAL OF INFECTION AND IMMUNITY, vol. 63, no. 3, March 1995 (1995-03), pages 884-890, XP000877152 cited in the application the whole document</p>	

